DEVELOPMENT AND APPLICATION OF ASSAYS FOR SEROTONIN

by

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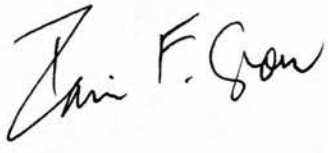
A thesis submitted for the degree of

Doctor of Philosophy, University of Edinburgh,

1987

DECLARATION

This thesis was composed by myself, and the work presented in it is my own. Measurements made by others in collaborative studies are as indicated in the acknowledgements and text.



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August 1987

Acknowledgements

I am grateful to my supervisors Dr J E T Corrie and Professor C R W Edwards for their expert advice and support throughout the duration of this work; in particular, I would like to thank Dr Corrie for the innumerable samples of blood he donated during the early developmental stages of the project. I would also like to thank Professor C R W Edwards for the use of the laboratory facilities in the University of Edinburgh Department of Medicine, Western General Hospital, Edinburgh. I am indebted to Dr P L Padfield for allowing me to use his patients and volunteers for the studies described in this thesis, and I wish to thank the research nurses Marie Reid, Jane Corrie, and Sue Stewart for the many venesections they performed on my behalf.

I also wish to thank the staff of the Department of Medicine, Western General Hospital, Edinburgh for carrying out the aldosterone and angiotensin II assays, and I am grateful to the staff of the Metabolic Unit, Western General Hospital, Edinburgh, for urinary sodium and potassium analyses. I am very grateful to the staff of the Animal Unit, Western General Hospital, Edinburgh, and in particular, Miss S Rossiter for maintaining and sampling the animals used in this thesis. I am grateful to the staff of the Haematology Department for performing platelet counts, and I am particularly grateful to Miss C Fiskerstrand and Mrs E Burnet for use of their aggregometer.

I would like to thank Mr P S Budd for his general advice, and his help with the sort routine in the BASIC program. Similarly, I am indebted to Dr B C Williams for allowing me to use his cannulated

rats, and I am also grateful for his criticism, support and enthusiasm, and proof-reading of this thesis. I would also like to thank Dr A Flappan for allowing me to study his heart failure patients, and also for his helpful criticism and general interest. I am grateful to Dr I Nimmo for his expert advice on analysis of the data from the uptake experiments.

I am very grateful to the Departmental secretaries Mrs G Proven and Mrs B Beattie for their tolerance during my occupation of the word-processors and photocopier. I am grateful to Mr K Miller for drawing some of the figures used in this thesis, and I am particularly grateful to my wife Dr Sadie M Gow for proof reading and criticism of the manuscript.

Dedication

This thesis is dedicated to the memory of my father, Mr James Gow.

Abstract

Abnormal levels of serotonin in blood have been described in several cardiovascular-associated diseases, but the role of this indoleamine in the establishment or progression of these diseases is not well understood. It is known that patients with essential hypertension have lower blood levels of serotonin, and there is some evidence to suggest reduced platelet uptake of serotonin. Angiotensin II (AII) has been shown to increase serotonin synthesis and to alter platelet aggregation; since AII is important in regulating sodium retention, the effect of sodium status on blood levels of serotonin and also platelet function required close examination. In this thesis, two assays for serotonin were developed, validated, and used to investigate the relationship between 1) platelet aggregation, serotonin levels and sodium status and 2) serotonin levels and platelet function in patients with cardiovascular disease.

A radioimmunoassay (RIA) using an [^{125}I]-labelled tracer was developed and validated for the measurement of serotonin in human platelet-rich plasma (PRP) and rat serum. Antisera were raised against N-succinamylserotonin conjugated to bovine albumin and, to improve assay sensitivity, the analyte was made chemically similar to the immunogen by conversion to N-acetylserotonin prior to assay, using the specific amino reagent N-acetoxysuccinimide. An assay for serotonin using high-pressure liquid chromatography with electrochemical detection (HPLC-ECD) was developed, and used to

validate the RIA. There was a good correlation between the RIA and HPLC-ECD reference method both for human PRP, and for rat serum, indicating that no significant cross-reactions were present, and that endogenous N-acetylserotonin concentrations were negligible compared with the levels of serotonin in human PRP and rat serum. The RIA can be used to assay up to 100 samples/day compared with 10-20/day by the HPLC-ECD assay.

Studies carried out in rats showed that an increase in sodium intake resulted in a decrease of serum serotonin; a similar effect was seen in normal human female PRP, although the difference between the serotonin levels was not significant when corrected for platelet number. In agreement with work by other groups, sodium loading resulted in an increase in platelet aggregation, although no effect on the rate of aggregation was found. This increase was blocked *in vitro* by the serotonin antagonist ketanserin, which also reduced the rate of aggregation. This suggests that the observed increase in the extent of aggregation may be mediated by enhanced release of serotonin from platelets during sodium loading. There was no correlation between plasma AII levels and serotonin concentration, although a correlation was seen between plasma AII levels and platelet aggregation.

The group of essential hypertensive patients studied in this thesis had lower levels of serotonin in their PRP compared with controls, but after correcting for platelet numbers, this difference was not significant. Radioactive impurities in the [^{3}H]-serotonin tracer used in the uptake studies contributed to the total observed count

rate, but not to the radioactivity incorporated by human PRP, and, if tracer purity had not been not corrected for, this would have resulted in a falsely depressed value for the amount of tracer taken up. There was no difference in the kinetics of serotonin uptake between the controls and the hypertensives.

In a group of patients with heart failure, chronic therapy with the angiotensin-converting enzyme inhibitor captopril resulted in an increase in PRP serotonin, possibly reflecting a prostaglandin-induced reduction of *in vivo* platelet aggregation.

The results suggest that the serotonin assays developed are useful for determining serotonin status in physiological or patho-physiological conditions, and may also indicate changes in platelet activation *in vivo*, since, unlike other markers of platelet activation (e.g. β-thromboglobulin), no special precautions have to be taken to minimise *ex vivo* platelet activation.

Abbreviations

A = amperes, current
AII = angiotensin II
AIII = angiotensin III
ACD = acid/citrate/dextrose anticoagulant
ACE = angiotensin-converting enzyme
ADP = adenosine diphosphate
APUD = amine precursor uptake and decarboxylation
AR = analytical grade reagent
BSA = bovine serum albumin
BTG = β-thromboglobulin
cAMP = cyclic adenosine monophosphate
Ci = curies
cpm = counts per minute
CSPPP = charcoal-stripped platelet-poor plasma
CV = coefficient of variation
DARS = donkey anti-rabbit serum
DMF = dimethylformamide
dpm = disintegrations per minute
EBSS = Earle's balanced salt solution
ECD = electrochemical detection
EDTA = ethylene diamine tetra-acetic acid
GPR = general purpose grade reagent
5HIAA = 5-hydroxyindoleacetic acid
HPLC = high-pressure liquid chromatography
HSA = human serum albumin
5HT = 5-hydroxytryptamine, serotonin
5HTP = 5-hydroxytryptophan
KHS = Krebs-Henseleit saline
K_a = affinity constant
K_d = dissociation constant
K_m = Michaelis constant
MAO = monoamine oxidase
MPV = mean platelet volume
5MT = 5-methoxytryptamine
NAD = nicotinamide adenine dinucleotide
NAS = N-acetoxysuccinimide
NIGPS = non-immune guinea pig serum
NIRS = non-immune rabbit serum
NSB = non-specific binding
NSHPP = N-succinimidyl-3-(4-hydroxyphenyl)-propionate
ODS = octadecyl silica
OPT = o-phthalaldehyde
PCPA = p-chlorophenylalanine
PPP = platelet-poor plasma
PRP = platelet-rich plasma
PVC = polyvinyl chloride
RBC = red blood cell
RCF = relative centrifugal force
REA = radioenzymatic assay

RIA = radioimmunoassay
SAGPS = sheep anti-guinea pig serum
SEM = standard error of the mean
SD = standard deviation
TLC = thin layer chromatography
TXA_2 = thromboxane A_2
UV = ultra-violet
V = volts, voltage
v_i = initial rate of uptake
V_{max} = maximum rate of uptake
WBC = white blood cell
$\bar{x}$ = arithmetic mean

Contents

Chapter 1

General Introduction

1.1 Introduction

It is nearly 85 years since Battelli (1905) described the vasoconstrictive properties of blood sera from various species of animals, and almost 40 years since the probable structure and biochemical properties of that serum vasoactive compound (serotonin) were first described (Rapport, et al., 1948a, 1948b). Even so, much remains to be discovered about the physiological and pathophysiological role of serotonin in man. This chapter is intended to serve as a general introduction to serotonin and its measurement; for more detailed reviews of serotonin, the reader is referred to, for example, Garrattini and Valzelli, 1965, and Smythe, 1979.

1.2 Discovery of Serotonin

Serotonin was discovered independently by two groups: following their studies on the enterochromaffin cells of the gastrointesinal tract (e.g. Vialli and Erspamer, 1933), Erspamer's group found a compound which was apparently specific to the gut. Rapport and colleagues (1947) isolated the compound which gave blood serum its vasoconstrictive properties, with the view that this may be important in essential hypertension (plasma was found to have virtually no vasoactivity). Erspamer's group named their putative hormone enteramine, whilst Rapport and his colleagues called their discovery serotonin, and described its possible structure and chemical properties (Rapport, et al., 1948b). Later work (Erspamer and Asero, 1952) confirmed that enteramine and serotonin were in fact the same compound, the indoleamine 5-hydroxytryptamine (5HT; more correctly named 5-hydroxy-3-(2-aminoethyl)-indole; Figure 1.1).

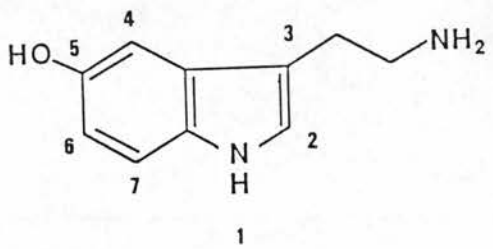


Figure 1.1 The structure of serotonin.

1.3 Biosynthesis and Metabolism of Serotonin

The major site of synthesis of serotonin in man is in the enterochromaffin cells of the gut (Erspamer and Testini, 1959). These cells (originally named because of the intense staining after dichromate fixation (Ciaccio, 1906)) have also been described as amine precursor uptake and decarboxylation, or APUD cells (Pearse, 1968). Serotonin is synthesised from dietary L-tryptophan via the intermediate compound 5-hydroxytryptophan (5HTP; Figure 1.2). This is not a major metabolic pathway for tryptophan, as only 1-2% of dietary tryptophan is converted into serotonin (Levine, 1974). Since the enzyme converting tryptophan to 5HTP is not saturated with substrate *in vivo* (Friedman, *et al.*, 1972), and since 5HTP is rapidly decarboxylated to serotonin (Tappaz and Pujol, 1980), it appears that the rate-limiting step in serotonin biosynthesis is the 5-hydroxylation of tryptophan by tryptophan hydroxylase (Lovenberg, *et al.*, 1967; Friedman, *et al.*, 1972). The hydroxylation of tryptophan into 5HTP requires the presence of a cofactor, a tetrahydrobiopterin (Figure 1.3), and it has been suggested that the concentration of the oxidised form of this cofactor (dihydrobiopterin) may also play a regulatory role in the rate of serotonin synthesis (see Boadle-Biber, 1982). However this seems unlikely since an increase in tetrahydrobiopterin concentrations *in vitro* did not lead to an increase in serotonin synthesis (Bullard, *et al.*, 1978).

The production of 5HTP from tryptophan by tryptophan hydroxylase is inhibited by *p*-chlorophenylalanine (PCPA; Koe and Weissman, 1966); this provides a specific method of inhibiting serotonin synthesis

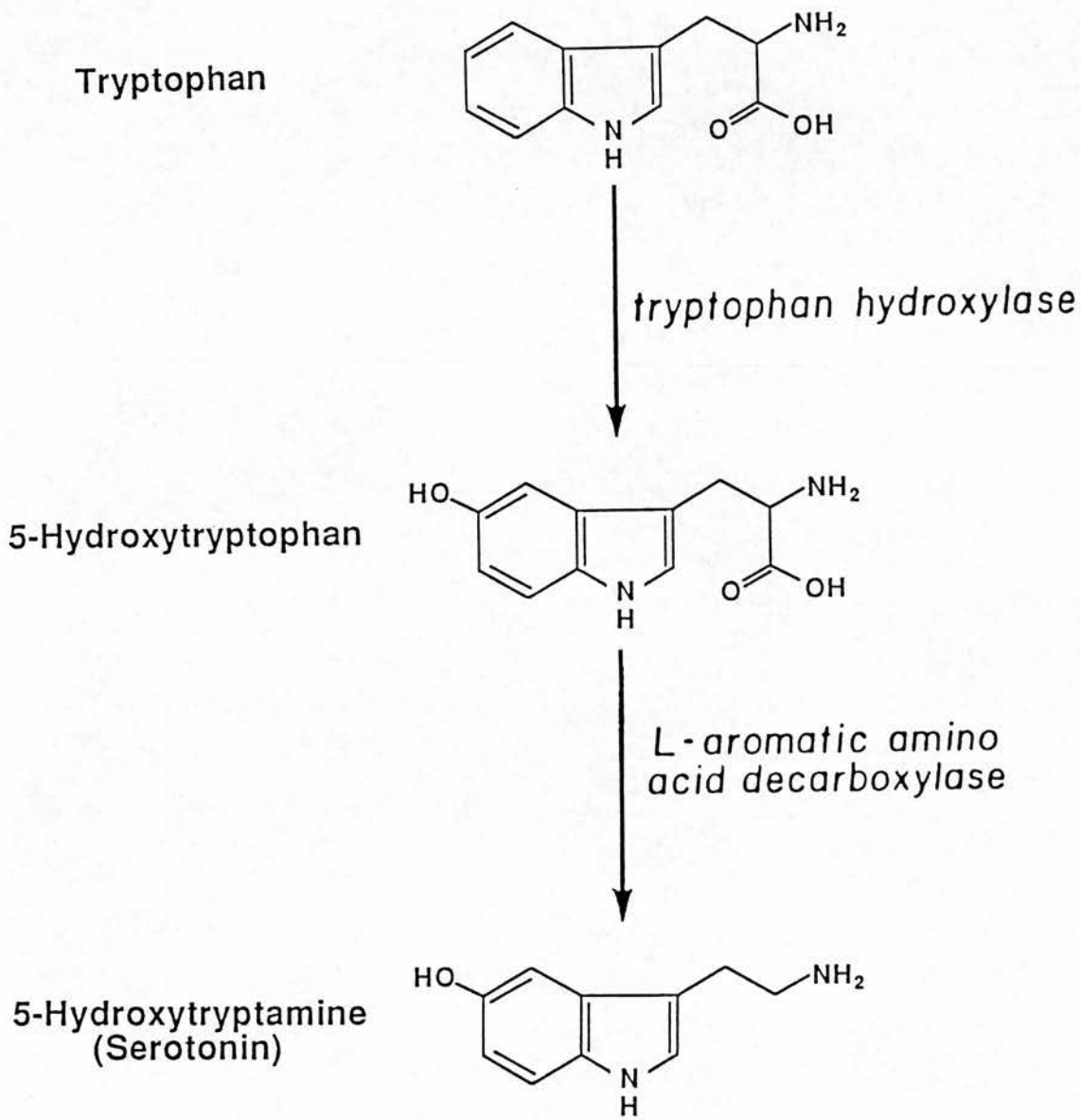


Figure 1.2 Biosynthesis of serotonin from tryptophan.

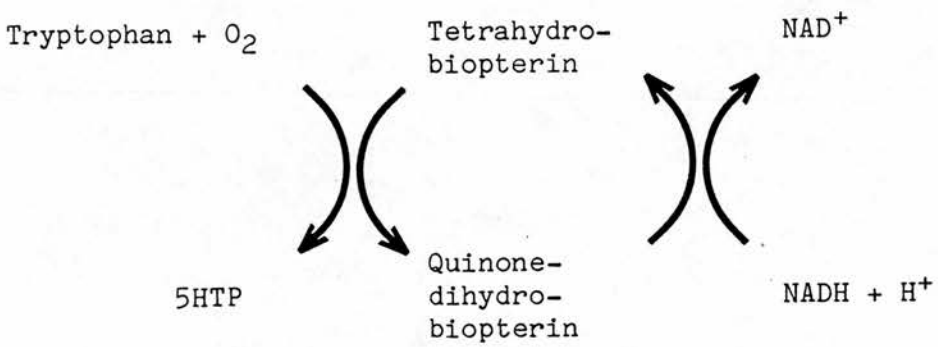


Figure 1.3 **The role of tetrahydrobiopterin in the hydroxylation of tryptophan.**

which can be used to investigate the effects of serotonin depletion (e.g. Jequier, et al., 1967). This is preferential to blocking serotonin synthesis by inhibition of the enzyme which converts 5HTP into serotonin (L-aromatic amino acid decarboxylase), since this decarboxylating enzyme is also required for the production of catecholamines (Lovenberg, et al., 1962). Once formed, serotonin can undergo several other reactions, the major pathways being 1) conversion into N-acetylserotonin, which can then be converted into 5-methoxy-N-acetyltryptamine (melatonin; Figure 1.4a), or 2) oxidation to 5-hydroxyindoleacetic acid (5HIAA) via 5-hydroxyindoleacetaldehyde (Figure 1.4b). Formation of melatonin from serotonin via N-acetylserotonin occurs primarily in the pineal gland (Pang and Tang, 1983), and is of particular interest since there is a marked rhythm in the levels of melatonin in blood, and also in the activity of the enzyme N-acetyltransferase, which is responsible for the synthesis of N-acetylserotonin from serotonin (Pang, et al., 1980). The peak of N-acetyltransferase activity is greatest during the hours of darkness, and coincides with the peak levels of serotonin in the pineal (see Sugden, et al., 1983).

Serotonin is oxidised to 5-hydroxyindoleacetaldehyde by monoamine oxidase type A which is located in cellular mitochondria (Pearce and Roth, 1983). Further oxidation by aldehyde dehydrogenase produces 5HIAA, large quantities of which are excreted in urine (up to 50 μmol/day; Udenfriend, et al., 1956). Serotonin is metabolised in this way in the liver (Drapanas and M^{c}Donald, 1963) and the lungs (Gillis, et al., 1979).

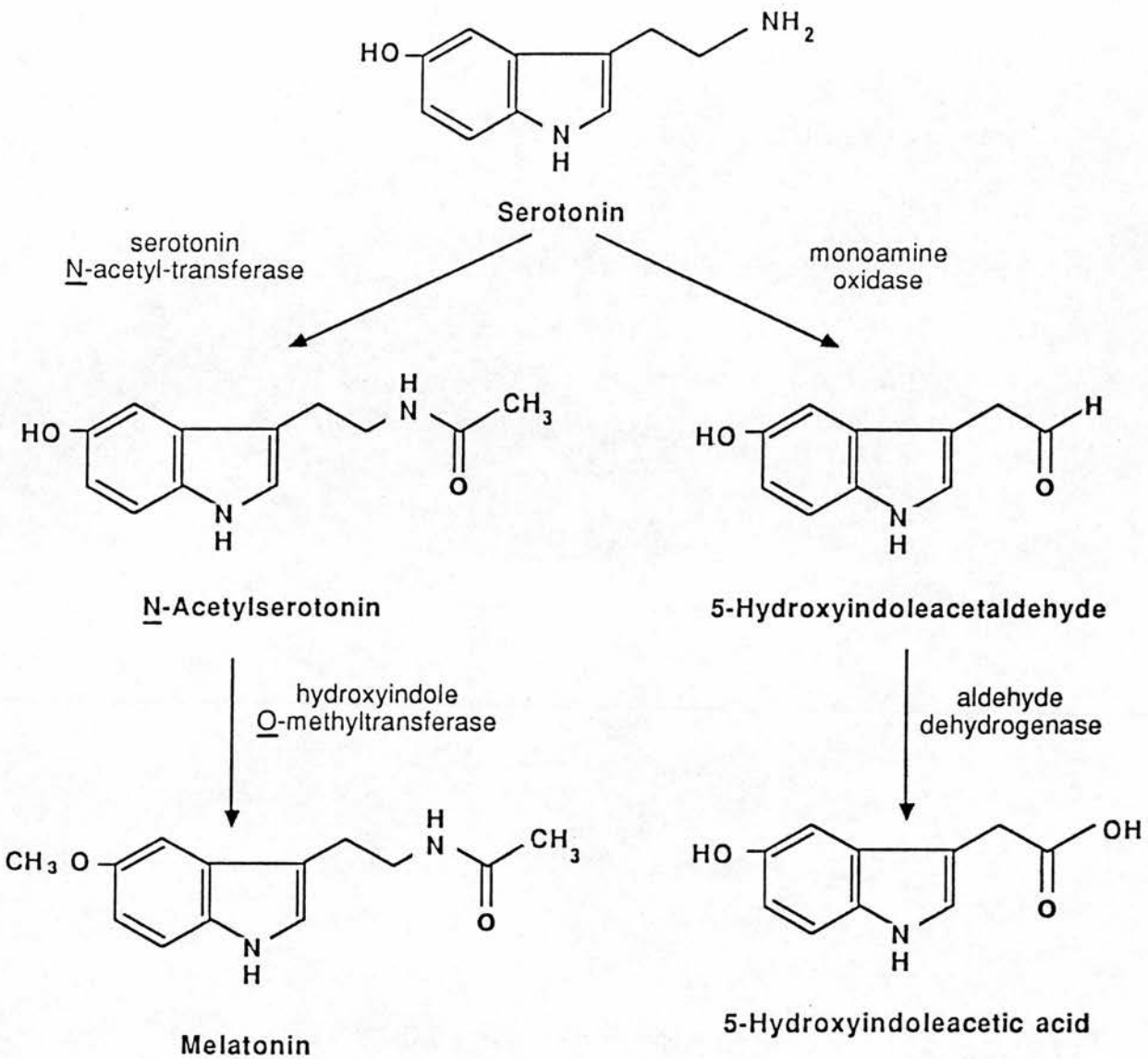


Figure 1.4 Metabolism of serotonin

a) conversion into melatonin

b) conversion into 5HIAA.

1.4 Serotonin and the Platelet

The major storage site for serotonin in human blood is the platelet (Rand and Reid, 1951; Udenfriend and Weissbach, 1954); however, in other mammals (e.g. the rat), serotonin is also found in mast cells (Benditt, et al., 1955). Platelets are small blood cells (diameter approximately 3 μm) with no nucleus, and therefore little capacity to synthesise protein. They are formed by fragmentation of the cytoplasm of megakaryocytes, which themselves derive from pluripotent stem cells in the bone marrow (Thompson, 1986). Platelets contain definite internal structures, (Figure 1.5; see White and Gerrard, 1978), notably dense granules which are the storage sites for serotonin (Davis and White, 1968), and α-granules which store the platelet-specific proteins β-thromboglobulin and platelet factor 4 (Day, et al., 1973). Serotonin is stored in the dense granules complexed with di- and tri-nucleotides (e.g. ADP, ATP) and heavy metal cations (e.g. Ca^{2+}, Mg^{2+}; see Pletscher, 1968).

Serotonin is released from the enterochromaffin cells after stimulation of the gut (e.g. by vagal stimulation; Ahlman and Dahlström, 1983) into the bloodstream, and is then available either for metabolism by the liver or the lungs, or uptake by platelets (Toh, 1954). Platelet serotonin uptake can be either a) active or b) passive:

a) active uptake is energy dependent, since it is inhibited by metabolic poisons (e.g. cyanide; Born and Gillson, 1959). Active uptake requires external Na^+ and Cl^- ions (Campbell and

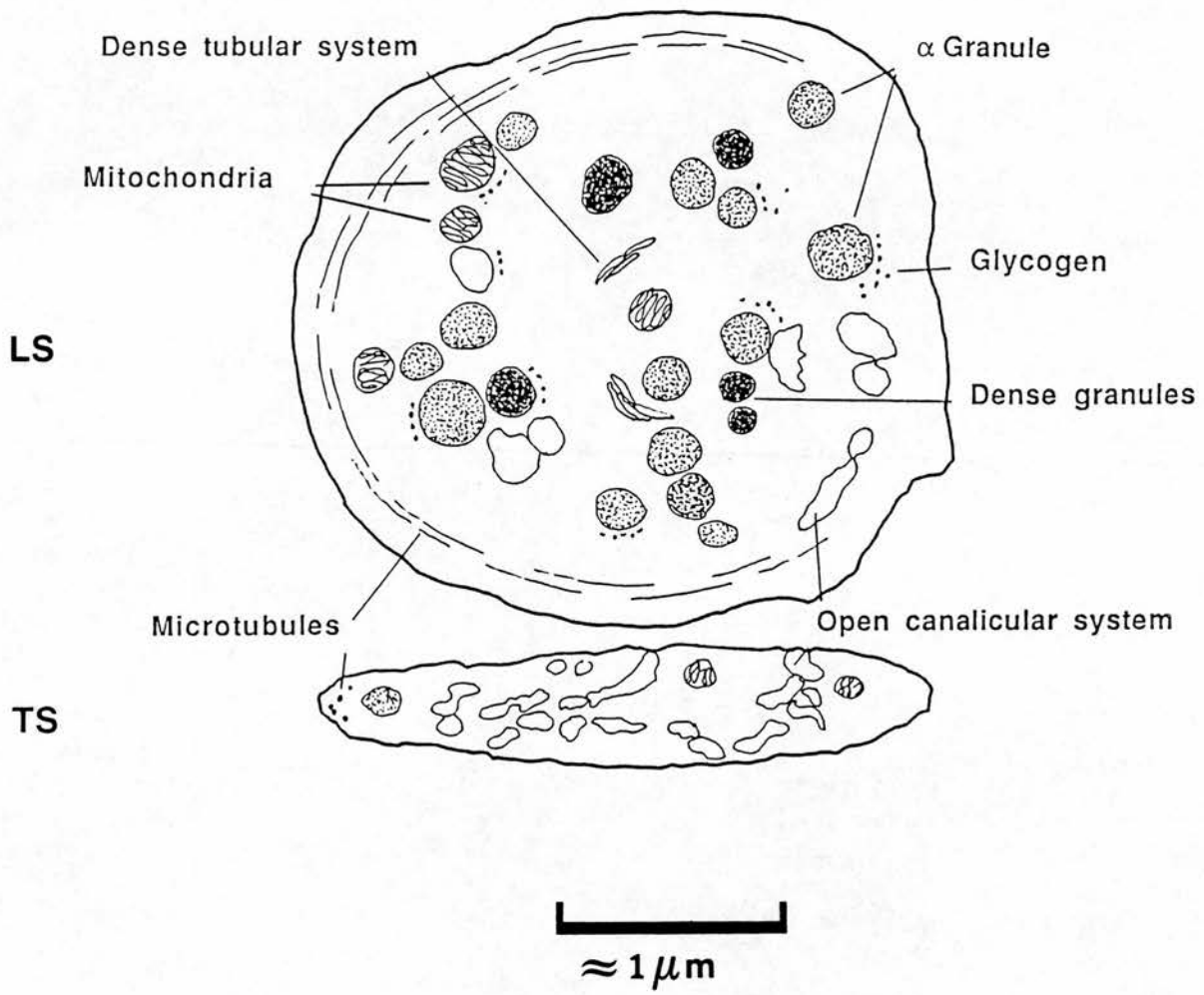


Figure 1.5 The internal structure of the platelet

LS = longitudinal section TS = transverse section.

Todrick, 1973; Nelson and Rudnick, 1982; Rudnick, et al., 1983), and internal K^+ ions; it is thought that serotonin is co-transported with a Na^+ and Cl^- ion, in exchange for a K^+ or H^+ ion (Nelson and Rudnick, 1982). Platelet serotonin receptors in man are of the $5HT_2$ type (De Clerck, et al., 1984), and are further divided into two sub-groups of 'A' and 'B' type receptors (Peters and Grahame-Smith, 1980). The 'A' receptor is of low capacity, but high affinity, and is thought to be involved in the serotonin-induced aggregation response of platelets whereas the 'B' receptor is of low affinity and high capacity, and participates in the active uptake of serotonin (Peters and Grahame-Smith, 1980). The 'B' receptor is inhibited non-competitively by the tricyclic antidepressant chlorimipramine (Lingjaerde, 1979), which reflects the similarities between synaptosomes (the presumed "active site" for tricyclic anti-depressants) and platelets (Sneddon, 1973). It has been suggested that the related antidepressant imipramine "straddles" two uptake sites, since studies have shown that binding of each molecule of [^{3}H]-imipramine requires two Na^+ ions (Rudnick, et al., 1983), compared with the 1:1 ratio seen between serotonin and Na^+ for uptake. Active serotonin uptake is rapid, saturable, and the initial rate is thought to follow Michaelis-Menten kinetics (e.g. Humphrey and Toh, 1954).

b) In contrast, passive uptake or diffusion is not dependent on cellular energy, but is the dominant uptake process at high

external concentrations of serotonin (Born and Bricknell, 1959). Passive uptake is temperature dependent, the rate declining with decreased temperature (Pletscher, et al., 1966).

Once internalised, serotonin is transported into the dense granules for storage. This process is unaffected by metabolic inhibitors, but can be blocked by reserpine (see Da Prada, et al., 1978). Osim and Wylie (1983) showed that stored platelet serotonin may be released and accumulated by other tissues. These workers labelled the platelet membranes with ^{111}Indium, and the platelet stores of serotonin with [^{14}C]-serotonin; they found in certain organs (notably the thyroid, skin and the adrenals) an increase in the $^{14}C/^{111}In$ ratio, suggesting preferential uptake of [^{14}C]-serotonin by these organs.

Serotonin is released from the platelet following stimulation by e.g. thrombin, which results in >90% of the total platelet serotonin being released (Grette, 1962). This is clearly seen in the clotting reaction (i.e. thrombin production and fibrin formation) of whole blood; the thrombin produced (Figure 1.6; for a review, see Vermylen, 1978) results in the release of platelet serotonin into the surrounding fluid, thus producing the serum-specific vasoconstrictor sought for and isolated by Rapport, et al., (1947). Plasma prepared by centrifugation of anticoagulated blood contains only a fraction of the serotonin concentration of serum, since thrombin formation is a calcium-dependent process, and anticoagulation of blood by calcium-chelating agents (e.g. EDTA, citrate) inhibits thrombin production, and hence platelet degranulation and release of serotonin. Thus

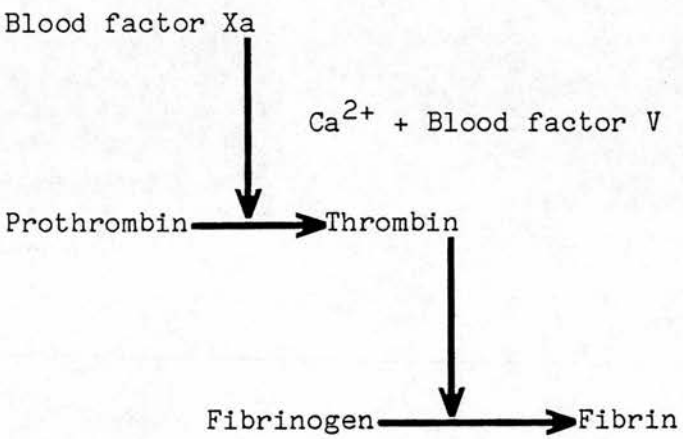


Figure 1.6 Production of thrombin during clotting of blood.

serum is rich in serotonin, whereas plasma is relatively serotonin-free. Release of serotonin from the dense granules occurs after the platelet has responded in other ways, e.g. by a shape change, or aggregation. In general, depending on the concentration and the strength of the stimulating agent, one or more of the platelet responses may be observed, but they always proceed in the order of: 1) shape change, 2) aggregation, and finally, 3) the release reaction (for a review, see Holmsen, 1979). It is thought that in the circulation in vivo, there are significant levels of released ("free") serotonin, which may contribute to various pathological states (see Gershon and Tamir, 1985); whether "free" serotonin is of biological importance, or whether it is an artifact of sample collection and processing remains to be conclusively established.

1.5 Measurement of Serotonin

Several different techniques have been used to measure serotonin in biological tissues and fluids, the most common of which are 1) bioassay, 2) fluorimetry, 3) radioenzymatic assay (REA), 4) high-pressure liquid chromatography (HPLC), and 5) radioimmunoassay (RIA). The approximate detection limits for these different assays are shown in Table 1.1.

1.5.1 Bioassay

Bioassays for serotonin rely on the ability of serotonin to produce a contractile response in smooth muscle cells (e.g. rat colon; Feldberg and Toh, 1953), and the degree of contraction is proportional to the amount of serotonin in the sample. This is a

Assay	Detection Limit (fmol)
Bioassay	570
Fluorimetry	30,000
Fluorimetry (OPT)	6,000
REA	100
HPLC	400
RIA	200

Table 1.1 Relative detection limit of different assays for serotonin (after Smythe, 1979).

sensitive method, but not specific, since other compounds present in the sample may also cause contraction of smooth muscle (e.g. histamine). Consequently, this type of assay is now rarely used to measure serotonin.

1.5.2 Fluorimetry

Serotonin fluoresces in strong acid with an emission at 550 nm (Udenfriend, et al., 1955) and this property has been used to measure serotonin concentration in e.g. brain tissue (Bogdanski, et al., 1956). The sensitivity of this method can be improved by derivatisation of serotonin with, for example, o-phthalaldehyde (OPT; Maickel and Miller, 1966). Other 5-hydroxyindoles also fluoresce however, and to improve the specificity of the method, serotonin should be extracted, for example by means of a cation-exchange resin (e.g. Amberlite CG-50), followed by washing and elution with acid or buffer (Awapara, et al., 1960).

1.5.3 Radioenzymatic Assay

Saavedra, et al., (1973) described the method of measuring serotonin after derivatisation into [^{3}H]-melatonin. The derivatisation as originally described was performed in two steps: i) serotonin was N-acetylated by incubation with N-acetyltransferase (purified from rat liver) and acetyl coenzyme A; this was followed by ii) [^{3}H]-methylation of the 5-hydroxy group of the N-acetylated serotonin (N-acetylserotonin) using hydroxyindole-O-methyltransferase (purified from bovine pineal) and [^{3}H-methyl]-S-adenosyl-L-methionine as the substrate. The [^{3}H]-melatonin produced in this way was

extracted into toluene, and counted directly in a liquid scintillation counter. The recovered count rate was directly proportional to the original concentration of serotonin and endogenous N-acetylserotonin in the sample. Hence in order to measure the serotonin concentration in tissues or fluids which contain appreciable amounts of N-acetylserotonin (e.g. the pineal gland; Pang and Tang, 1983), the endogenous N-acetylserotonin concentration must also be measured by including blanks which contain no acetylating enzyme. The true value for serotonin is then calculated by subtracting the unacetylated blank from the apparent total concentration of serotonin (Saavedra, et al., 1973).

A major disadvantage with this assay is that the enzyme required for the acetylation step degrades rapidly, and also contains some decarboxylase activity (Saavedra, 1977), which may result in production of serotonin from endogenous 5HTP in the sample, leading to falsely elevated results. To circumvent this problem, other workers have used N-acetyltransferase purified from the fruit fly, Drosophila melanogaster, since this enzyme is reported to be more stable, and lacks decarboxylase activity (Walker, et al., 1983). Alternatively, chemical N-acetylation using acetic anhydride has been described (Hammel, et al., 1978; Hindberg, 1984). Although the REA is a sensitive technique (detection limit typically 100 fmol/tube; Hammel, et al., 1978), the tritiated substrate is expensive, the methyltransferase enzyme is not readily available, and a multi-step procedure followed by liquid scintillation counting precludes throughput of large numbers of samples.

1.5.4 High-pressure Liquid Chromatography

The basic principle of reversed-phase HPLC is that a low-volume sample of analyte (5-500 µl) is injected into a stream of a carrier liquid (mobile phase) which is being pumped under high-pressure (1-4,000 psi) and at a constant flow rate through a steel column packed with silica particles (stationary phase). Covalently linked to these particles are hydrophobic aliphatic carbon chains, usually C_8 or C_{18} in length, and the greater the length of the carbon chain, the greater is the degree of hydrophobicity (Mefford, 1985). These carbon chains will interact with analytes dissolved in the mobile phase, the extent of interaction depending on hydrophobic strength of the analyte molecule. This is an intinsic property of the analyte, and different molecular species are thus retained to different degrees, i.e. have different characteristic retention times.

In reversed-phase chromatography, polar (hydrophilic) compounds elute from the column fastest, and non-polar (hydrophobic) compounds are retained longer. The separation can be made more selective by use of an ion-pairing agent (Horvath, et al., 1977), an amphipathic compound (e.g. octane-1-sulphonic acid) with a hydrophobic "head", and hydrophilic electrically charged "tail" (octane-1-sulphonic acid has an overall negative charge when ionised). The "head" of the molecule interacts with the C_{18} groups bonded to the silica, leaving the charged "tail" free to interact with compounds dissolved in the mobile phase. The addition of octane-1-sulphonic acid therefore effectively creates a cation-exchange column, which retards the

elution of hydrophilic cations (e.g. serotonin, which has a positively charged amino group at and below physiological pH (Sakurai and Ishimitsu, 1975)) and thereby increases their retention time.

After the compound of interest have been separated by the chromatographic column, they must be detected as they elute. The fact that serotonin can readily be oxidised (see Garattini and Valzelli, 1965) is utilised in the electrochemical detector (ECD): as the serotonin molecules elute from the column, they flow through a cell across which a fixed voltage is continuously being applied. The potential difference causes electrons to be removed from the serotonin molecules, changing them to quinone-imine structures (Mefford, 1981), and the resulting flow of electrons per unit time (i.e. current) is directly proportional to the number of serotonin molecules passing through the cell. This current is amplified and recorded as a peak, the area and height of which is proportional to the mass of oxidised analyte. Since different compounds oxidise to varying extents at different applied potentials, this can be used to increase post-separation selectivity. Furthermore, comparison of the oxidation profiles obtained by injection of a fixed mass of standard compounds or unknown material at different applied potentials under otherwise fixed chromatographic conditions (oxidative voltammetry) can be used to further identify unknown peaks (see also section 3.2.4).

After standardising the HPLC-ECD system with a known mass of serotonin, unknowns can be injected and concentrations calculated from simple comparision of peak heights or areas. The former is

more commonly used, as the height can be measured directly; the latter method necessitates the inclusion of an integrator into the HPLC system, increasing the capital outlay.

In summary, HPLC-ECD can be used to identify and quantitate an unknown peak by comparision, with a pure standard, of retention times, peak heights for a constant injected mass at different applied voltages (oxidative voltammetry), and co-elution of the unknown and standard peaks on co-injection.

1.5.5 Radioimmunoassay

The underlying principle of a competitive RIA is that antibodies raised against an analyte will bind that analyte, or a radioactive derivative (i.e. tracer). This tracer will compete with the unlabelled ("cold") analyte for the available antibody binding sites, and consequently will be displaced from the antibody in a dose-dependent manner, by increasing concentrations of "cold" analyte (assuming a fixed mass of tracer, and fixed concentration of antibody are present). The proportion of bound or unbound (free) tracer to the total amount of tracer added is constant for a given mass of cold analyte under identical experimental conditions. At equilibrium, the relationship obeys the law of mass action (Ekins, 1974):

$$Ag + Ab \underset{k_2}{\overset{k_1}{\rightleftharpoons}} Ag.Ab$$

where Ag is the analyte, Ab the antibody, and Ag.Ab the analyte/antibody complex (it is assumed that the tracer behaves in a similar manner to the native analyte). The forward reaction rate (association rate) is k_1, and the backward reaction (dissociation

rate) is k_2. The affinity constant of the reaction (K_a) is given by the ratio of k_1 to k_2; thus the greater the association rate, the greater the affinity constant, i.e. the greater the avidity of the antibody. The dissociation constant (K_d) is the ratio of k_2 to k_1, i.e. the reciprocal of K_a. The displacement of bound tracer resulting from the incubation of unknown samples of analyte with a fixed mass of tracer and antibody is compared with that given by several standard masses of pure analyte under the same conditions, hence the mass of analyte in the unknown can be calculated. Thus for an RIA system there are four basic requirements:

i) an antibody raised against the compound to be studied which has a high specificity and avidity for (i.e. binds strongly) the analyte,

ii) a tracer of high specific activity which can be bound by the antibody, but displaced by cold analyte,

iii) a pure standard analyte, and

iv) a means of separating the bound tracer from the free without perturbing the established equilibrium.

1.5.5.1 Production of Antibody Containing Plasma or Serum

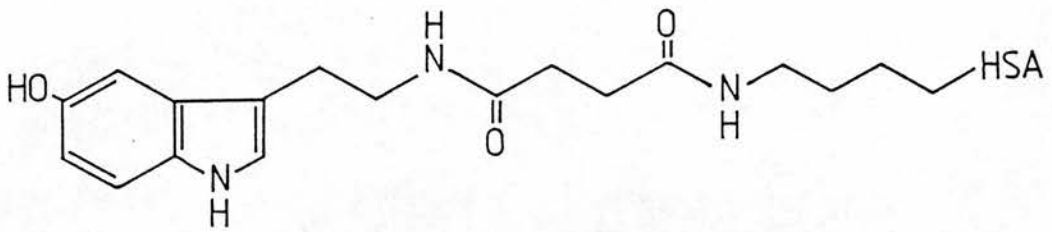
Immunisation of experimental animals with serotonin itself will not evoke an immune response, since i) serotonin is present in the blood of the animal as an endogenous substance, and therefore will not be seen as "foreign" by the host's immune system, and ii) it is too small (molecular weight 176) to elicit an immune response, the smallest immunogens having molecular weights >1000.

These two problems are classically solved by conjugation of several molecules of the analyte to a large carrier protein molecule from an animal species different to that used for raising the antibodies (for a review, see Erlanger, 1980). This conjugation effectively increases the size of the analyte (or hapten, as the conjugated form of the analyte is termed) and also renders it "foreign"; antibodies will be directed against antigenic regions (epitopes) of the conjugate, some of which will include the hapten-protein complexes and the bridge structures linking the two molecules. Antibodies directed against these epitopes retain a degree of avidity for the unconjugated analyte, and it is this property which enables these antibodies to be used for immunoassay.

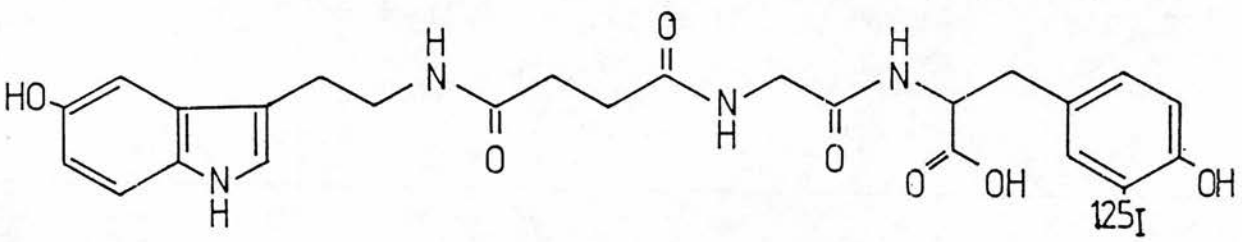
Antibodies to serotonin for RIA were raised some fourteen years ago by Peskar and Spector (1973). These workers conjugated serotonin through the 6 position of the indole ring by diazotisation of D,L-p-aminophenylalanine conjugated to bovine serum albumin, and used rabbits for immunisation. The antibodies were harvested in platelet-poor plasma (PPP), since rabbit platelets have a high serotonin content (Garattini and Valzelli, 1965), most of which is released from platelets during clotting, and would otherwise interfere in the assay by saturation of the antibody binding sites. Even after taking such precautions, the antibodies obtained from PPP could only be used at a dilution of 1/50 (this gave 30% binding of total added tracer), compared with 1/10 for serum. The antibodies were also found to cross-react strongly (93%) with 5-methoxy-tryptamine (5MT).

Other groups (Kellum and Jaffe, 1976, Engbaek and Voldby, 1982) used the same methodology for the preparation of the immunogen, and collected the antibodies from rabbits either as PPP (Kellum and Jaffe, 1976) or serum, which was then dialysed against activated charcoal to remove endogenous serotonin prior to use (Engbaek and Voldby, 1982). Again, the working dilutions of "antiserum" used were low (<1/500), and there was a high cross-reaction with 5MT (>25%). However, since the levels of 5MT in plasma are normally low relative to platelet serotonin (Franzen and Gross, 1965), this cross-reaction may only interfer with the assay significantly when measuring non-platelet-bound serotonin. The tracer used by all the above groups was tritiated serotonin.

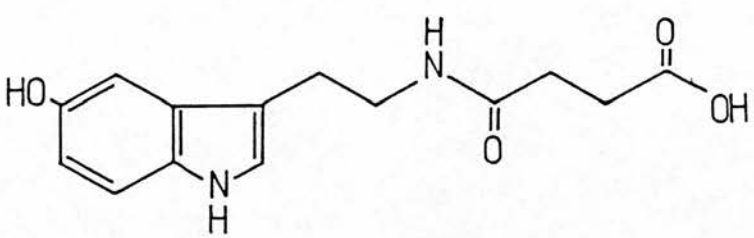
A different approach was used by Delaage and Puizillout in 1981 (with later modification (Geffard, et al., 1982)). It had already been demonstrated that antibodies raised against cyclic adenosine monophosphate (cAMP) using a 2'-O-succinyl-cAMP-serum albumin conjugate had greater avidity for 2'-O-succinyl-cAMP than for cAMP itself (Cailla, et al., 1973). Fransden and Krishna (1976) later showed that the 2'-O-succinylation step could be replaced by 2'-O-acetylation for cAMP. This principle of chemically modifying the analyte to resemble the original immunogen more closely (Figure 1.7) was used by Delaage and Puizillout in their RIA for serotonin: these workers raised antibodies to the N-succinamyl derivative of serotonin conjugated to human serum albumin (HSA), and samples/standards were succinylated at the 5-hydroxy group and N-terminal by treatment with succinic anhydride. Prior to assay, the 5-O-succinyl group was



N-SUCCINAMYLSEROTONIN-HSA



N-SUCCINAMYLSEROTONIN-GLYCYL-[^{125}I]-TYROSINE



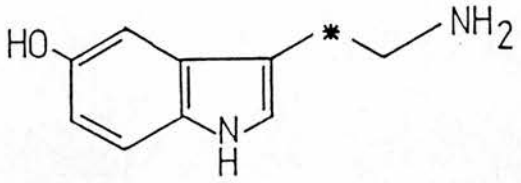
N-SUCCINAMYLSEROTONIN

Figure 1.7 **Immunogen, tracer and modified analyte as used in the serotonin RIA of Delaage and Puizillout (1981).**

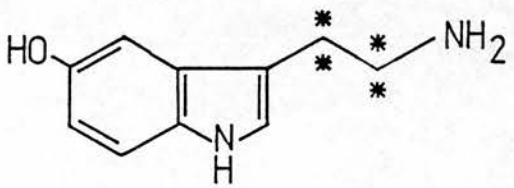
hydrolysed back to a hydroxy group by brief exposure to alkali, since the disuccinyl form would be expected to be bound less well by the antibody than the N-succinamyl derivative (Cailla, et al., 1973). In the modified assay (Geffard, et al., 1982) antibodies were raised against 5-methoxy-N-succinamyltryptamine-HSA, and the samples of serotonin were chemically acetylated and methylated to 5-methoxy-N-acetyltryptamine (melatonin) prior to assay. Since the 5-hydroxy group is methylated, it is protected from acetylation, and consequently no alkali hydrolysis step is required. This assay required an organic extraction step, but did illustrate that the N-acetyl derivative of serotonin could be used instead of the N-succinamyl form to displace tracer bound to the antibody. In both these acylation assays, the working dilution of antibody was >1/10,000, and an [^{125}I]-tracer was used. The antibody was collected as serum, since unmodified serotonin was ineffective in displacing the tracer, and therefore need not be removed.

1.5.5.2 Tracers Used in Serotonin Radioimmunoassays

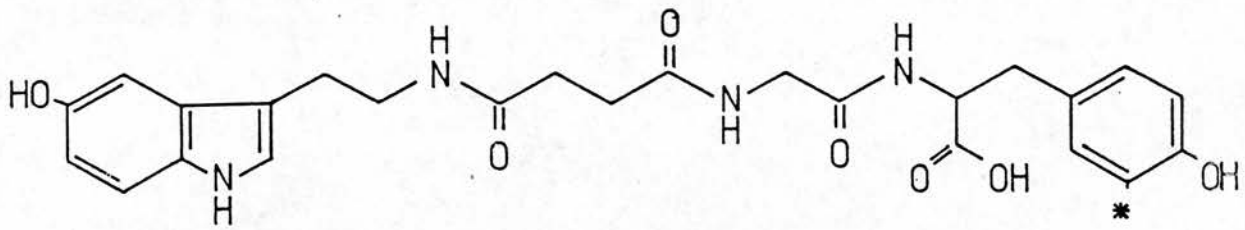
Tritiated serotonin (Figure 1.8) has been used extensively as a tracer in serotonin RIAs (Peskar and Spector, 1973; Kellum and Jaffe, 1976; Engbaek and Voldby, 1982). This β-emitting nuclide is chosen in preference to ^{14}C due to its higher specific activity (29 Ci/mmol compared with 0.062 Ci/mmol respectively for the ^{14}C form), i.e. the count rate of tritium is approximately 500 times that of ^{14}C, mol/mol. Pure (>95%) tritiated serotonin is readily available commercially, and, due to the longer half-life (12.3 years compared with 60.2 days for ^{125}I), theoretically has a longer shelf life than



[side chain ^{14}C]-serotonin



[side chain ^{3}H]-serotonin



N-succinamylserotonin-glycyl-[^{125}I]-tyrosine

* = site of substitution

Figure 1.8 Radioactive derivatives of serotonin.

the [^{125}I]-labelled derivative of serotonin, but the latter has a higher specific activity (and therefore count rate) compared with the tritiated tracer (2,200 Ci/mmol, and 29 Ci/mmol respectively, assuming 100% isotopic abundance and one radioactive atom per molecule). Furthermore, ^{125}I tracers may have variable effects on assay parameters, but careful attention to the chemistry of the tracer and assay design can often lead to enhanced performance over that available from ^{3}H tracers (Corrie and Hunter, 1981).

The gamma emissions from the decay of ^{125}I can be detected directly (i.e. without sample pre-treatment), unlike β-emitting tracers, which require an addition of liquid scintillant, which is time consuming, costly, and may introduce further errors into the assay. A γ-emmitting tracer is therefore highly desirable for an assay intended for routine use, with a throughput of large numbers of samples.

Unlike tritium or ^{14}C, ^{125}I cannot be substituted for an existing stable atom in most biological molecules, the exception being the thyroid hormones, where iodine is an intrinsic component of the hormone. Consequently, a method of coupling ^{125}I to the analyte must be found. With large molecules (e.g. angiotensin II, AII), ^{125}I can be substituted for a hydrogen in tyrosine, histamine, or tyramine groups with little loss (e.g. 25%) in biological activity, as has been demonstrated by specific binding of [^{125}I]-AII to cellular AII receptors (Lin and Goodfriend, 1970). Smaller molecules which do not contain any of these groups can be conjugated to histamine, tyramine, or their derivatives in a manner similar to

the preparation of the immunogen, and then iodinated (e.g. Geffard, et al., 1982). Alternatively, to minimise damage to the analyte by the oxidising and reducing agents used during the iodination and avoid inclusion of the large ^{125}I atom in the analyte molecule with subsequent loss of immunoreactivity, the histamine or tyramine derivative can be iodinated and then conjugated to the analyte (Nars and Hunter, 1973). Similarly, analytes containing free amino groups can be iodinated by the elegant technique of Bolton and Hunter (1972): these workers used N-succinimidyl-[^{125}I]-(3-iodo-4-hydroxyphenyl)-propionate to specifically acylate amino groups in proteins, but the technique is equally applicable to any compound that contains a free amino group. In the serotonin RIAs which used ^{125}I-tracers, [^{125}I]-glycyl tyrosine was conjugated to either N-succinamylserotonin (Delaage and Puizillout, 1981), or 5-methoxy-N-succinamylserotonin (Geffard, et al., 1982). In the former method, the glycyl tyrosine was iodinated prior to conjugation, which avoided the destruction of the 5-hydroxyindole nucleus by the oxidising agent (chloramine T) used to introduce ^{125}I into the tyrosine ring. The second method was a direct iodination of the tracer molecule, since the methoxy group at the 5 position on the indole ring prevented oxidation. The tracers prepared by both these methods are reported to have high specific activities (approximately 2,000 Ci/mmol), and were stable when stored as aliquots (diluted in buffer) at -20°C for 4-5 months.

1.5.5.3 Standard Serotonin

Standard serotonin is usually prepared commercially as either the creatinine sulphate, or the hydrochloride salt. The creatinine sulphate salt is most widely used, since it is cheaper, and reputedly more stable than the hydrochloride (Garattini and Valzelli, 1965). The purity of commercially available serotonin is >99%.

1.5.5.4 Methods of Separation

Three methods of separating free tracer from the bound fraction have been used in previous RIAs for serotonin:

1) precipitation with 50% saturated ammonium sulphate (Peskar and Spector, (1973), Kellum and Jaffe, (1976)),
2) equilibrium dialysis (Delaage and Puizillout, (1981), Geffard, et al., (1982)), and
3) liquid-phase second-antibody precipitation (Engbaek and Voldby, 1982).

1. Ammonium Sulphate Precipitation

Precipitation by ammonium sulphate is based on the principle that immunoglobulins have low solubilities, and will therefore precipitate out at concentrations of precipitating reagents that should leave other polypeptides in solution (Thorell and Larson, 1978). This method is markedly affected by the protein concentration in the plasma/serum sample, as the immunoglobulins contained therein will also be precipitated, thus increasing the bulk of the pellet, and trapping free tracer, which will then be misclassified as bound (Ratcliffe, 1974).

2. Equilibrium Dialysis

As a method of separating free analyte from bound, equilibrium dialysis is not widely used; indeed, it appears to be restricted to a single research group (i.e. Delaage and Puizillout, 1981; Geffard, et al., 1982). Separation is achieved as follows: the incubation of antibody, analyte, and tracer is carried out in a cuvette which is bisected by a semi-permeable membrane, with all the reagents in buffer on one side, and buffer only on the other. A membrane is used with a pore size which will only allow the passage of tracer which is not bound to the antibody; at equilibrium, equal aliquots are removed from both sides of the cuvette and counted. The aliquot from the side with all the reagents corresponds to the bound and free fractions, whereas the aliquot from the buffer side is a measurement of the free only; the relative ratios of bound to free ligand can then be calculated. This system allows only small numbers of samples to be assayed, which negates one of the fundamental advantages of radioimmunoassay, namely the throughput of large numbers of samples (Ratcliffe, 1974). Perhaps the only advantage of equilibrium dialysis is that no centrifugation step is required, and hence no significant perturbation of the equilibrium should occur.

3. Liquid-phase Second Antibody Separation

This separation system uses an antiserum ("second" antibody) raised against antibodies from the same species in which the anti-analyte antibodies (i.e. "first", or primary antibody) were raised (Morgan and Lazarow, 1963). Generally, the second antibody is added

to the pre-incubated first antibody/antigen/labelled antigen mixture, followed by a period of further incubation and centrifugation of the resulting immunoprecipitate, thus leaving the bound fraction of tracer in the pellet, and the unbound, or free fraction in the supernatant, which can easily be decanted or aspirated to waste. Carrier serum from an animal (the same species as the first antibody) which has not been immunised is added prior to the second antibody to provide sufficient immunoreactive material to form a lattice which can be centrifuged down (Hunter, 1978). The optimum concentrations of first antibody, second antibody, and carrier serum which provide maximal recovery of specifically bound tracer in the bound fraction are determined by incubating a fixed dilution of first antibody with varying dilutions of second antibody and carrier serum (Hunter and Ganguli, 1971). Alternative methods of second antibody (also known as double antibody) separation exist, e.g. where the first antibody, carrier serum, and second antibody are pre-incubated to form a pre-precipitate, which after washing to remove remaining free first antibody, can be added directly to the analyte/tracer mixture (e.g. Gray, et al., 1983). Addition of a single aliquot of a pre-precipitate avoids inclusion of imprecision resulting from the addition of first antibody, carrier serum, and second antibody as single aliquots.

Charcoal separation, although not used previously in other RIAs for serotonin, has been used successfully for other hapten RIAs (e.g aldosterone; Al-Dujaili and Edwards, 1981). Unlike second antibody separation, however, charcoal separation can be sensitive to time,

temperature, pH and serum protein concentration (Ratcliffe, 1974; Thorell and Larson, 1978; Binoux and Odell, 1973), and can sometimes result in a significant misclassification of "free" vs. bound radioligand.

1.6 Serotonin in Pathological Situations

Abnormal blood levels of serotonin have been described in many diseases including a) depression, b) migraine, c) essential hypertension, and d) the carcinoid syndrome.

a) Depression. It has been suggested that the diminished levels of serotonin and decreased uptake into platelets of patients with depression (Tuomisto and Tukiainen, 1976) may indicate a defect in neurotransmission. The similarity between platelets and synaptosomes is reflected in the effectiveness of tricyclic antidepressants as inhibitors of the active transport of serotonin into platelets, and platelets therefore offer a practical way to monitor the efficacy of such drugs (Tuomisto, 1976). Also, lithium, which is used prophylactically in depression, has been shown to normalise the uptake of serotonin into the platelets of depressed patients in vitro (Murphy, et al., 1969).

b) Migraine. Migraine sufferers are known to exhibit several serotonin-associated disorders during, and for several hours after an attack: the blood content of serotonin is reduced (e.g. Hilton and Cumings, 1972), the platelets show an impaired ability to take up serotonin when incubated in endogenous plasma, but not when incubated in a plasma-free medium, or in plasma from a normal volunteer

(Launay, et al., 1982; Pradalier and Launay, 1982). Furthermore, the excretion of the serotonin metabolite 5HIAA increases during an attack (Gawel, et al., 1979), and plasma prepared from migraineurs during an attack markedly stimulates the release reaction of normal platelets compared with plasma from the same patients outwith an attack, or plasma from normal volunteers (Mück-Seler, et al., 1982). Although it seems clear that serotonin and platelets are involved in the pathology of migraine, much is unknown about the mechanisms which result in the observed abnormalities of serotonin biochemistry and platelet function. Hence clarification of the role of serotonin in migraine may ultimately lead to better management of this disease.

c) Essential hypertension. Decreased levels of blood serotonin have been reported in patients with essential hypertension, and the uptake of serotonin into platelets from patients with essential hypertension is also reduced (e.g. Bhargava, et al., 1979; Kamal, et al., 1984a). Also, the release of [^{3}H]-serotonin from platelets prepared from patients with essential hypertension can be induced with lower concentrations of thrombin compared with platelets taken from normotensive controls (Valtier, et al., 1986). Bhargava, et al. (1979) suggested that the impairment of platelet serotonin uptake in patients with essential hypertension reflected a neural control defect, which becomes expressed as an elevation of blood pressure. However, it would seem that the overall view of platelet function in patients with essential hypertension suggests enhanced platelet activation in vivo, which would explain many of the reported effects

(for a review, see De Clerck, 1986). It should be borne in mind that some controversy still exists in this area, and some groups (e.g. Ahtee, et al., 1974; Feltkamp, et al., 1984) have found no differences in platelet serotonin content or platelet serotonin uptake kinetics between patients with essential hypertension and normal controls.

The effectiveness of the serotonin antagonist ketanserin in lowering blood pressure in patients with essential hypertension (De Cree, et al., 1981; Wenting, et al., 1984) suggests that serotonin may be an important factor at least in the maintenance, if not the development of the disease. Thus an understanding of the role of serotonin in this disease may ultimately be important in establishing better clinical management of these patients.

d) The carcinoid syndrome. This disease is possibly unique, in that it is the only situation where measurement of blood levels of serotonin, or urinary levels of the metabolite 5HIAA are used as part of the clinical diagnosis (Gitlow, et al., 1972). In the carcinoid syndrome, blood levels of serotonin are elevated (Pernow and Waldenström, 1957), and there is an increase in the urinary excretion of the serotonin metabolite 5HIAA (Page, et al., 1955), due to overproduction of serotonin by a tumour which synthesises serotonin from dietary tryptophan, via 5HTP. Up to 60% of dietary tryptophan can be metabolised in this way in the carcinoid syndrome, compared with the normal convertion ratio of 1% (Udenfriend, et al., 1956); this in turn may lead to diseases associated with a deficiency of dietary tryptophan, such as pellagra-type skin diseases (Zarafonetis,

et al., 1958), or mental disorders (Lehmann, 1966). The carcinoid tumour which gives rise to the syndrome typically occurs in the small intestine, and derives from the enterochromaffin cells of the gut (for a review, see Grahame-Smith, 1977).

A patient with the carcinoid syndrome usually has the clinical features of facial flushing, breathlessness, diarrhoea, and heart lesions (Thorsen, 1958). These symptoms only present when either the primary tumour is in the liver and drains directly into the bloodstream, or if liver metastases are present, since otherwise the active compounds secreted by the tumour (e.g. serotonin or kallikrein) are inactivated by a normal liver (see Grahame-Smith, 1977). It seems likely that the elevated circulating levels of serotonin present in the carcinoid syndrome may cause the diarrhoea, since treatment with serotonin antagonists (e.g. methysergide or ketanserin; Peart and Robertson, 1961; Ahlman, et al., 1984) or an inhibitor of serotonin synthesis (i.e. PCPA; Engelman, et al., 1967) have some beneficial effect in the loss of water and electrolytes. The high blood levels of serotonin may also be responsible for the cardiac lesions, since these usually occur on the side of the heart exposed to the highest concentrations of serotonin (i.e. the right side), and the left side is protected to some extent by metabolism of serotonin during passage of the blood through the lungs (Gillis, et al., 1979).

In summary, although serotonin was characterised nearly half a century ago, very little is known about its role in physiology or pathophysiology. This may be attributed partly to the intrinsic

instability of the molecule, and also partly due to the lack of assays suitable for screening studies, or for throughput of large numbers of experimental samples. The aim of this thesis, therefore, was to develop and validate assays for serotonin, and show that these assays could be applied to the study of serotonin in various physiological and pathophysiological conditions associated with cardiovascular diseases.

Chapter 2

Materials and Equipment

2 Materials and Equipment

2.1 Reagents

2.1.1 Indoleamines

N-Acetylserotonin	Sigma Chemical Co., Ltd., Poole, Dorset, England
5-Hydroxyindole-3-acetic acid	" " " "
5-Hydroxytryptamine creatinine sulphate	" " " "
5-Hydroxy-L-tryptophan	" " " "
5-Methoxytryptamine	" " " "
Tryptamine	" " " "
L-Tryptophan	" " " "
Nω-Methylserotonin	Aldrich Chemicals, Poole, Dorset, England
N-Succinamylserotonin	Gift from Dr J E T Corrie
O,N-Diacetylserotonin	" " " "

2.1.2 HPLC

Methanol (HPLC grade)	Rathburn Chemicals Ltd., Walkerburn, Peebles, Scotland
Water (HPLC grade)	" " "
Sodium dihydrogen orthophosphate (AR)	BDH Chemicals, Ltd., Glasgow, Scotland
Ethylenediaminetetra-acetic acid disodium salt (EDTA; AR)	" " "

Octane-1-sulphonic acid (sodium salt)	Sigma Chemical Co., Ltd., Poole, Dorset, England

2.1.3 Buffers/diluents

Citric acid	Sigma Chemical Co., Ltd., Poole, Dorset, England
L-Ascorbic acid	" " " "
L-Cysteine (hydrochloride)	" " " "
Bovine serum albumin	Armour Pharmaceutical Co., Ltd., Eastbourne, Sussex, England
Boric acid (AR)	BDH Chemicals Ltd., Glasgow, Scotland
Calcium chloride (AR)	" " "
Magnesium sulphate (AR)	" " "
Potassium chloride (AR)	" " "
Potassium dihydrogen orthophosphate (AR)	" " "
Sodium bicarbonate (AR)	" " "
Sodium hydroxide (AR)	" " "
Gelatine	Fisons Scientific Apparatus, Loughborough, Leicester, England
Sodium chloride (AR)	" " "
Sodium acetate trihydrate	Koch-Light Laboratories, Ltd., Bucks, England
Glacial acetic acid	May and Baker, Dagenham, England

Perchloric acid	May and Baker, Dagenham, England

2.1.4 Anticoagulants, etc.

D(+)Glucose (dextrose)	Sigma Chemical Co., Ltd., Poole, Dorset, England
Trisodium citrate	" " " "
o-Phenanthroline	" " " "

2.1.5 Acylation

Succinic anhydride (GPR)	BDH Chemicals, Glasgow, Scotland
Triethylamine (AR)	" " "
Acetic anhydride	Fisons Scientific Apparatus, Loughborough, Leicester, England
Glycine	Sigma Chemical Co., Ltd., Poole, Dorset, England
Potassium hydroxide	May and Baker, Dagenham, England
N-Acetoxysuccinimide	Gift from Dr J E T Corrie

2.1.6 β-emitting tracers, and purification reagents etc.

5-Hydroxy-[side chain ^{3}H]-tryptamine creatinine sulphate (specific activity 20-50 Ci/mmol)	Amersham International PLC, Amersham, England
Amberlite CG-50 (100-200 wet mesh)	Sigma Chemical Co., Ltd., Poole, Dorset, England
Three-way luer tap	AHS International, Herstal, Belgium
Glass wool	BDH Chemicals, Glasgow, Scotland

Polypropylene syringe (1 ml)	Becton Dickinson, Dun Laoghaire, Co. Dublin, Ireland
Scintran "Cocktail T" (liquid scintillant)	BDH Chemicals, Glasgow, Scotland

2.1.7 γ-emitting tracers, and preparative reagents etc.

Sodium ^{125}iodide (specific activity 1,650-2,157 Ci/mmol)	Amersham International, PLC, Amersham, England
Potassium iodide (AR)	BDH Chemicals, Glasgow, Scotland
N-Chloro-p-toluene-sulphonamide (chloramine T)	" " "
Sodium metabisulphite (AR)	" " "
Ethyl acetate (AR)	" " "
Dimethylformamide (AR)	" " "
N-succinimidyl-3-(4-hydroxy-phenyl)-propionate (NSHPP)	Sigma Chemical Co., Ltd., Poole, Dorset, England
Histamine (free base)	" " " "
Glycyl tyrosine	" " " "
Isobutyl chloroformate	" " " "
Tri-n-butylamine	Aldrich Chemical Co., Poole, Dorset, England
Sephadex G-25	Pharmacia Fine Chemicals, Milton Keynes, England

2.1.8 Assay Separation Systems

Ammonium sulphate (Grade I)	Sigma Chemical Co., Ltd., Poole, Dorset, England
Activated charcoal (250 - 350 mesh)	" " " "
Dextran T-70	Pharmacia Fine Chemicals, Milton Keynes, England
Non-immune guinea-pig serum (NIGPS)	Scottish Antibody Production Unit Carluke, Lanarkshire, Scotland
Sheep anti-guinea-pig serum (SAGPS)	" " "
Non-immune rabbit serum (NIRS)	" " "
Donkey anti-rabbit serum (DARS)	" " "

2.1.9 Platelet Serotonin Uptake and Aggregation Studies etc.

Normal saline	Steripak, Runcorn, Cheshire, England
Chlorimipramine	Ciba-Geigy, Duxford, Cambridge England
Adenosine 5'-diphosphate (potassium salt)	Sigma Chemical Co., Ltd., Poole, Dorset, England
Thrombin (bovine plasma)	" " " "
p-Chlorophenylalanine	" " " "
Ketanserin	Janssen Pharmaceuticals, Ltd, Oxford, England
Saralasin (Sar^1-ala^8-angiotensin II)	Universal Biologicals, Cambridge, England
Medium 199 (solid)	Gibco Scientific, Paisley, Renfrew, Scotland

2.1.10 Miscellaneous

Anaesthetic ether	May and Baker, Dagenham, England
Captopril	E R Squibb, and Sons, Hounslow, Middlesex, England
Frusemide	Hoechst UK, Ltd., Hounslow Middlesex, England
CRM rat food	Labsure, Poole, Dorset, England
Oxalic acid (diammonium salt)	Sigma Chemical Co., Ltd., Poole, Dorset, England
Pargyline (hydrochloride)	" " " "
Amberlite XAD-5	" " " "
Sephadex G-50	Pharmacia Fine Chemicals, Milton Keynes, England
Freund's adjuvant	Difco Laboratories, Detroit, Michigan, USA

2.2 Equipment

2.2.1 HPLC-ECD

M6000A solvent delivery system	Waters Associates, Northwich, Chesire, England
U6K injector unit	" " "
LC-4A amperometric detector	" " "
UV detector (model 441)	" " "
Waters Data Module	" " "
Solvent clarification kit	" " "

Durapore filters (0.22 μm)	Waters Associates, Northwich, Chesire, England
C_{18} μBondapak columns (3.9 mm x 30 cm; 10 μm packing)	" " "
C_{18} Corasil Pre-column (3.9 mm x 2 cm; 37-50 μm packing)	" " "
C_{18} "Sep-paks"	" " "
ODS-Hypersil column (3.9 mm x 15 cm; 5 μm packing)	Capital HPLC, Sighthill, Edinburgh, Scotland
Hamilton syringe (25 μl)	V A Howe, London, England

2.2.2 Tubes, etc.

LP3 polystyrene tubes (10 mm x 75 mm)	Luckham, Ltd., Sussex, England
LP4 polystyrene tubes (12 mm x 75 mm)	" "
Pyrex borosilicate tubes (10 mm and 12 mm x 75 mm)	Corning Glass Works, Corning, New York, USA
Stoppered glass tube ("Quickfit", 10 ml)	Macfarlane Robson, Ltd., Glasgow, Scotland
Polypropylene microfuge tube (1.5 ml conical)	" " "
Polystyrene containers (2, 10, 25, 60, and 120 ml)	Sterilin, Ltd., Middlesex, England
Potassium EDTA tubes (5 ml)	" " "
Polystyrene aggregometer tubes (2 ml)	Teklab (ML), Ltd., Sacriston, Durham, England

2.2.3 Pipettes, etc.

Oxford pipettes:	10 - 50 µl	BCL, Ltd., Lewes, Sussex,
	50 - 200 µl	England
	200 - 1000 µl	
	fixed 50 µl	
	fixed 100 µl	
Pipette tips:	10 - 200 µl	" " "
	200 - 1000 µl	
Glass pipettes (5 and 10 ml)		Macfarlane Robson, Ltd., Glasgow, Scotland
Glass measuring cylinders (50, 100, 200, and 1000 ml)		" " "
Glass beakers (50, 100, 250, and 1000 ml)		" " "
Glass volumetric flasks (5, 10, 50, 100 ml)		" " "
Disposable transfer pipettes (polypropylene, 2 ml)		LIP, Shipley, Yorkshire, England

2.2.4 Iodinations

Peristaltic pump	LKB Instruments, South Croyden Surrey, England
"Ultrorac" fraction collecter (model 7000)	" " "
Chromatography column and fittings (49 x 1 cm)	Pharmacia Fine Chemicals, Milton Keynes, England
"Speedivac" vacuum pump	Edwards High Vacuum, Crawley Sussex, England
Glass chromatography tank	Macfarlane Robson, Ltd., Glasgow, Scotland
"Polygram" silica gel TLC plate (20 x 20 cm, 0.25 mm silica)	Camlab, Cambridge, England
X-ray film	Kodak, Manchester, England

2.2.5 Radioactivity Counting Equipment, etc.

"Tricarb" liquid scintillation spectrometer (model 3330) (counting efficiency 30%)	Canberra-Packard, Pangbourne, Berks, England
NE1612 "turbo" gamma counter and software (counting efficiency 87%)	Nuclear Enterprises, Sighthill Edinburgh, Scotland

2.2.6 Other Equipment

"Coolspin" centrifuge	MSE (Fisons) Crawley, Sussex, England
Refrigerated microcentrifuge	Camlab, Cambridge, England
Gallenkamp "Junior" bench centrifuge	Gallenkamp, Loughborough, Leicester, England
UV spectrophotometer (model SP8-100)	Pye-Unicam, Ltd., Cambridge, England
Coulter counter (Model ZB)	Coulter Electronics
Aggregometer (six channel) and chart recorders	Malin Electronics, Ayr, Scotland
Stir bars (polypropylene coated, 3 x 1 mm)	" " "
Hotplate stirrer (model PC351)	Mackay and Lynn, Ltd., Edinburgh, Scotland
"Rotamixer De-Luxe" vortex stirrer	Hook and Tucker, Ltd., England
"Oxford" dispenser (0.8 - 3.0 ml)	BCL, Ltd., Lewes, Sussex, England
Water-bath	Grant Instruments, Cambridge, England
Shaking water-bath	" " "
pH meter (Pye model 290)	Pye-Unicam, Ltd, Cambridge, England

Haemocytometer (improved Neubauer)	Macfarlane Robson, Ltd., Glasgow, Scotland
Needles (19 gauge)	Becton Dickinson, Dun Laoghaire, Co. Dublin, Ireland
Syringes (polypropylene, 5, 20, 50 ml)	" " " "
"Copal" electronic sphygmomanometer (model UA251)	Andrew Stephens and Co., Blackpool, England
Arterial cannulae (PVC, ID 0.4 mm, OD 0.8 mm)	Whitefield (Medical), Ltd., Edinburgh, Scotland
Sampling cannulae (PVC, ID 0.5 mm, OD 1.5 mm)	Altec, Alton, Hampshire, England
Catheter (French size 8)	Cordis Corporation, Miami, Florida, USA
95% O_2:5% CO_2	BOC, Brentford, Essex, England

2.3 Data Analysis

BBC Model B microcomputer	Acorn Computers, Ltd., Cambridge, England
Statistics package ($p<0.05$ taken to be statistically significant)	C S Hetherington, Newcastle, England

Chapter 3

Development of Assay Methods for Serotonin

3.1 Introduction

As outlined in the general introduction, serotonin can be measured by high-pressure liquid chromatography with electrochemical detection (HPLC-ECD), radioenzymatic assay (REA), or radioimmunoassay (RIA); HPLC-ECD is currently the most common method in use, having been applied to a wide range of biological materials, including whole blood (Korpi, 1984), serum (Sasa, et al., 1978), plasma (Tagari, et al., 1984), cerebrospinal fluid (Petrucelli, et al., 1982), brain tissue (Falkowski and Wei, 1981), platelets (Fujimori, et al., 1982) and adrenals (Verhofstad and Jonsson, 1983). Many published methods exist for the measurement of serotonin by HPLC, but most are similar with respect to the chromatographic and detection systems used.

Several radioimmunoassay (RIA) methods have been published, but only one was validated against an established reference method (Kellum and Jaffe, 1976); this assay suffered from two major disadvantages: firstly, only a low dilution of antibody could be used (<1/500), and secondly, tritiated serotonin was used as the tracer. In this thesis, an HPLC-ECD method was established, and used a) during the development and b) for the validation of an improved RIA technique for serotonin.

3.2 Development and Validation of an HPLC-ECD assay for Serotonin

3.2.1 Chromatographic Conditions for HPLC-ECD

The HPLC-ECD system used in this thesis was optimised to separate (in order of elution) 5-hydroxytryptophan, 5-hydroxy-indoleacetic acid, N-acetylserotonin, serotonin, and Nω-methyl-

serotonin. Several basic experiments were carried out to validate the HPLC-ECD system:

a) elution profile of freshly supplied radioactive serotonin,

b) detector response to a range of analyte concentrations with constant chromatographic conditions, and

c) detector response to fixed masses of different analytes, at different applied potentials, under otherwise constant chromatographic conditions.

The chromatographic conditions used were (unless otherwise stated) as follows: the mobile phase (pH 4.8) consisted of sodium di-hydrogen phosphate (5.4 g/l), octane-1-sulphonic acid (60-75 mg/l), EDTA (380 mg/l), methanol (15-20% v/v), and water (85-80% v/v). Prior to use, the mobile phase was filtered under reduced pressure through a 0.22 μm Durapore filter. Chromatographic columns used were stainless steel packed with octadecyl silica, and were either 30 cm x 3.9 mm with 10 μm particle size, or 10 cm x 3.9 mm with 5 μm particle size. The analytical column was protected by a pre-column of stainless steel (2 cm x 3.9 mm) packed with 37-50 μm diameter octadecyl silica. Analysis was performed at room temperature. Unless otherwise stated, the electrochemical detector was set at an applied potential of +0.60 V, with a sensitivity of 5 nA full scale deflection. The analytical electrode was polished glassy carbon, and the reference electrode was silver/silver chloride. Flow rate was 0.5-2.0 ml/min, and injection volumes were 10-1,000 μl, into a 2 ml sample loop. Stock standards were made up in a solution of perchloric acid/cysteine, pH 1.8 (150 and 0.2 mmol/l respectively),

and stored in glass in the dark at 4°C. The concentration of each standard was confirmed by UV analysis, using the formula:

$$A=\xi.c.l$$

where A is the absorbance, ξ is the extinction coefficient (l/mol.cm), c the concentration (mol/l), and l the path length (cm). The standard was used if the UV concentration was within 5% of the value calculated by weight. The extinction coefficient was taken to be 5740 l/mol.cm for each 5-hydroxyindoleamine, at a wavelength of approximately 275 nm, which was an absorption maximum for serotonin (Figure 3.1).

Serotonin was found to be acceptably stable when stored as described. When compared by HPLC-ECD analysis with a freshly prepared solution of serotonin, a solution which had been prepared and used over a period of nine months was found to contain 93.0% of the original serotonin concentration. The separation given by the system for a test mixture of (in order of elution) 5-hydroxy-tryptophan, 5-hydroxyindoleacetic acid, N-acetylserotonin, serotonin, and N_{ω}-methylserotonin is shown in Figure 3.2. Each compound was well resolved, and serotonin was separated from its immediate precursor (5-hydroxytryptophan) and the major serotonin metabolite (5-hydroxyindoleacetic acid).

3.2.2 Elution Profile of a Pure Radioactive Standard

Freshly supplied [side-chain ^{3}H]-serotonin (quoted purity >95%) was diluted 1/1,000 in a solution of perchloric acid/cysteine and an aliquot (50 µl, 50 nCi) co-injected with an aliquot (25 µl) of a

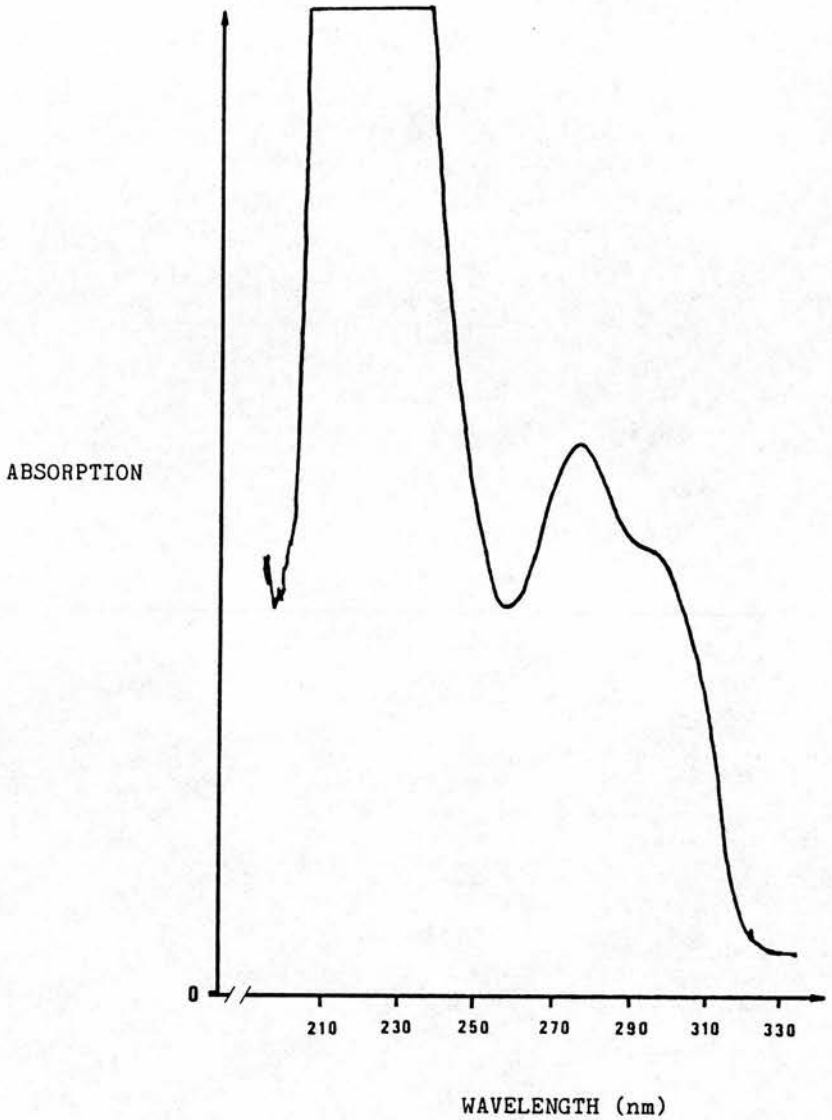


Figure 3.1 **Ultra-violet absorption spectrum of serotonin creatinine sulphate, dissolved in perchloric acid/cysteine (pH 1.8).**

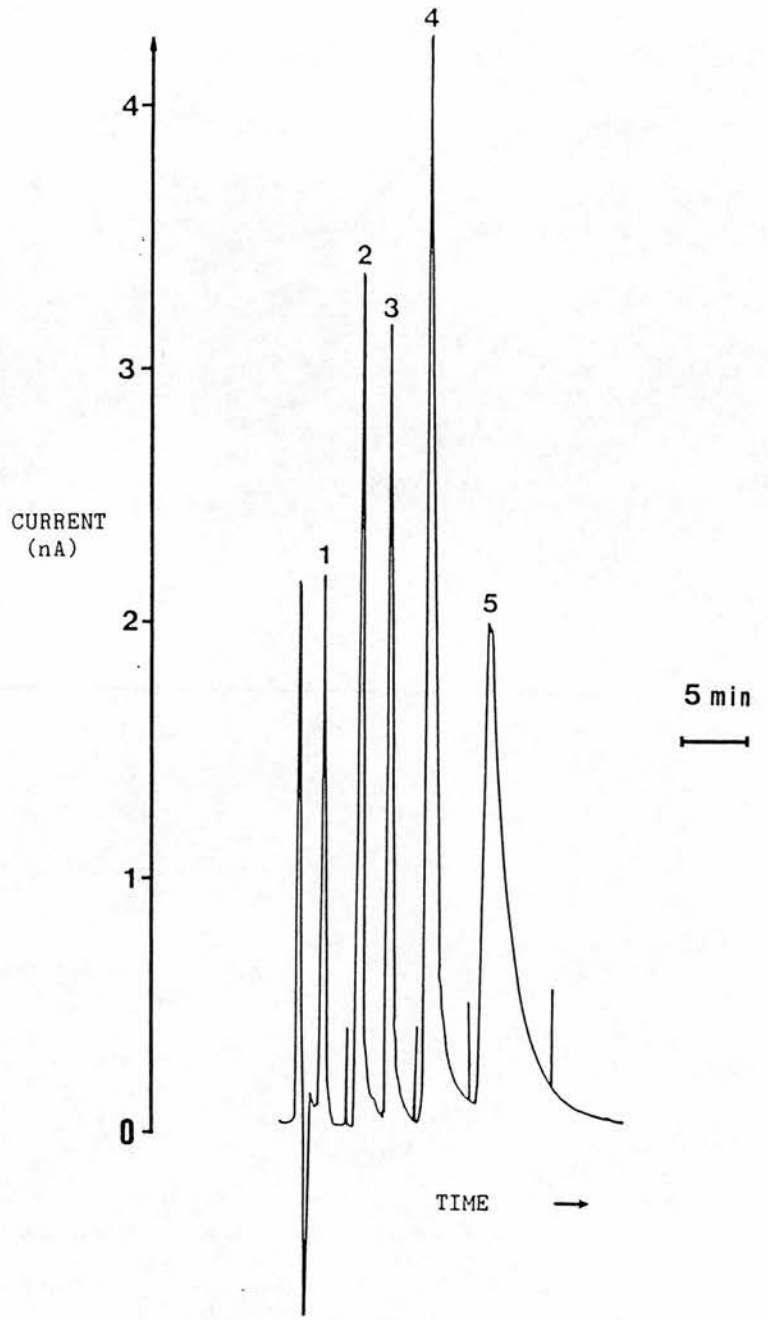


Figure 3.2 **Typical elution profile of serotonin and related indoleamines obtained using the HPLC-ECD system as described in the text:**

1 = 5-hydroxytryptophan (5HTP)
2 = 5-hydroxyindoleacetic acid (5HIAA)
3 = N-acetylserotonin
4 = serotonin
5 = Nω-methylserotonin (internal standard).

solution of standard serotonin in perchloric acid/cysteine (1 μmol/l).

After passing through the detector, fractions (1 ml) of the eluate from the column were collected every minute into polypropylene liquid scintillation vials. Total counts (in duplicate) consisted of mobile phase (1 ml) and tracer (50 μl); duplicate background samples were mobile phase only (1 ml). After the final fraction had been collected, liquid scintillant (10 ml) was added to each vial, the vials were capped, shaken vigorously for 30 s and then counted. The percentage radioactivity in each fraction was calculated as follows:

$$\frac{[\text{Sample} - \text{background}] \times 100}{[\text{Total} - \text{background}]}$$

A typical elution profile of [^{3}H]-serotonin from the HPLC-ECD is shown in Figure 3.3. As can be seen in comparison with the trace from the HPLC-ECD recorder, the peak of radioactivity was isographic with a peak of electrochemically active material eluting from the column.

3.2.3 Detector Response to Different Masses of Serotonin

A solution of standard serotonin was made up in acid/cysteine, and diluted to give a range of concentrations from 16 nmol/l to 50,000 nmol/l, in steps of five-fold increases in concentration. These solutions were stored at 4°C, and aliquots (50 μl) injected into the HPLC, each concentration being analysed three times. To avoid injection carry-over, the series of solutions was assayed starting with the most dilute, and progressing to the most

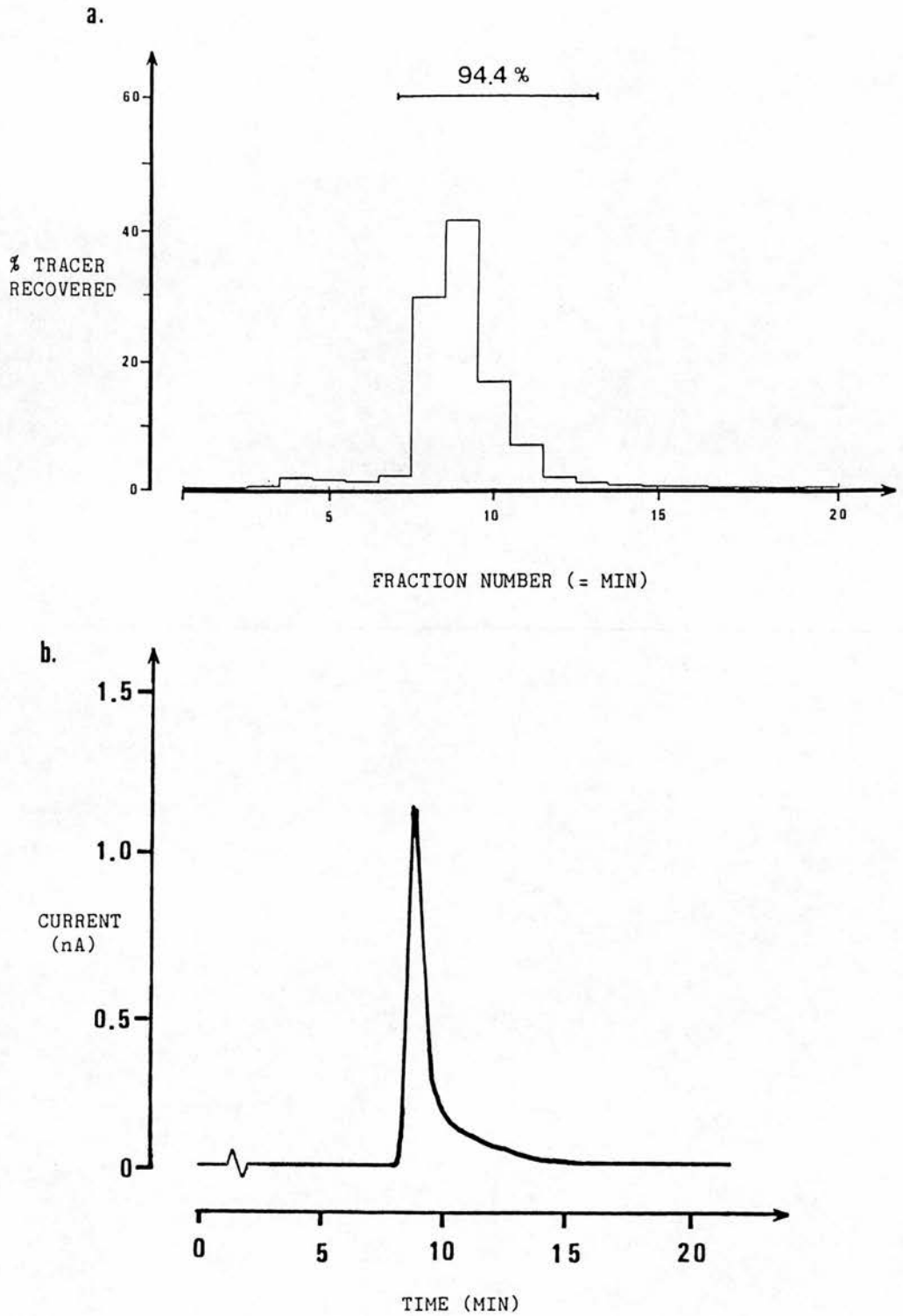


Figure 3.3 Elution profile of radioactivity from a) an injection of [^{3}H]-serotonin compared with b) the HPLC-ECD trace of a standard, non-radioactive solution.

concentrated. The sensitivity of the detector was initially 1 nA full scale, but was adjusted when necessary to keep peaks within the range of the chart recorder.

For calculation of results, peak heights (mm) were measured directly from the chart with a ruler, and the heights expressed relative to a sensitivity of 1 nA full-scale deflection. The detector response (as measured by peak height) to increasing serotonin concentration (Figure 3.4) was linear over the range tested (16-50,000 nmol/l), which is much greater than the range expected in most samples.

3.2.4 Detector Response to Different 5-Hydroxyindoleamine Derivatives at Different Applied Potentials

A mixture of 5-hydroxytryptophan, 5-hydroxyindoleacetic acid, N-acetylserotonin, serotonin, and N_{ω}-methylserotonin in acid/cysteine was analysed by HPLC-ECD with the detector set at different oxidation potentials. Flow rate was 1 ml/min, sensitivity was 5 nA full-scale deflection, and the volume injected was 25 µl each time. Three separate injections of the mixture were made at an initial applied voltage of +0.70 V, and the mean peak heights for each compound at this potential were assigned the value of 100%. The potential was decreased by 0.05 V prior to each subsequent injection (25 µl) of the mixture, which was stored at 4°C between injections. At the end of the experiment, the potential was returned to +0.70 V, and an injection of the mixture made, to confirm that the observed decreases in peak height were not due to degradation of the compounds occurring

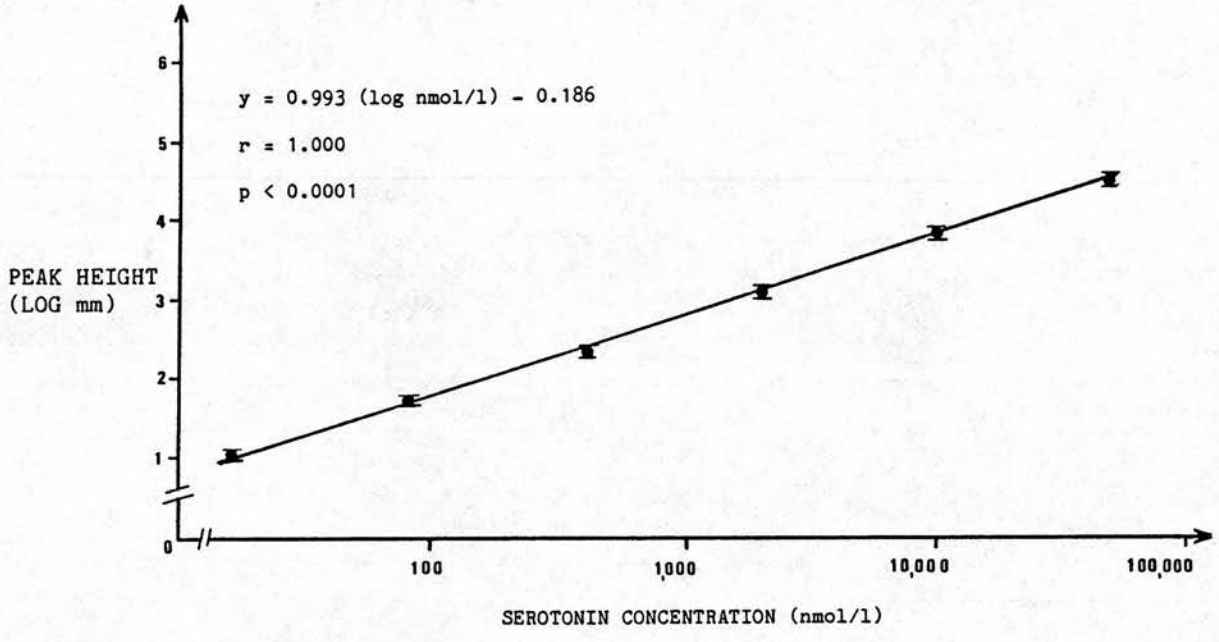


Figure 3.4 Detector response (as measured by peak height) to injection of different masses of standard serotonin; mean value ± SD of triplicate injections.

during the course of the experiment. Peak heights were measured, and expressed as percentages of the original injections at +0.70 V. Oxidative voltammograms were constructed by plotting applied potential vs. percentage peak height for each compound.

The response of the detector to different indoleamines at different applied potentials is shown in Figure 3.5. All the tested indoleamines gave an oxidation profile different to that obtained with standard serotonin, indicating that this technique may be used to confirm the identity of sample peaks eluting from the HPLC-ECD.

3.2.5 Preparation of Human Platelet-rich and Platelet-poor Plasma (PRP and PPP) for Serotonin Assays

Whole blood was taken from the antecubital vein (after applying a tourniquet) using a 19 gauge needle, and added to the anti-coagulant, which was acid/citrate/dextrose, ACD, (citric acid (8 g/l), trisodium citrate (22 g/l), glucose (20 g/l) in 10 ml plain polystyrene tubes; 9 vol blood:1 vol anticoagulant). The citrated whole blood was then used to prepare either citrated PRP or PPP:

a) PRP. The blood was centrifuged immediately at room temperature for 10 min at 121 x g, and the upper three-quarters of the supernatant (PRP) harvested with a disposable transfer pipette. An aliquot (500 µl) was added to a standard potassium EDTA tube (5 ml) and sent to the Haematology Department, Western General Hospital, for platelet counts; the remainder was stored frozen at -20°C.

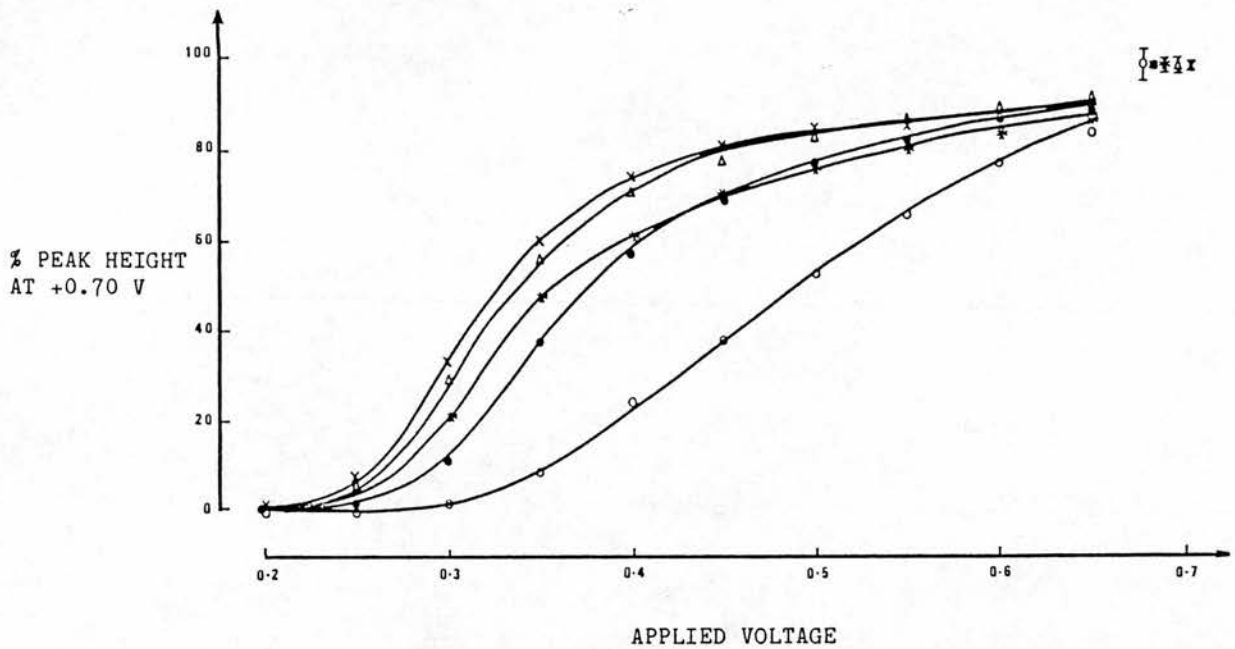


Figure 3.5 Detector response to different indoleamines at different applied oxidation potentials:

● = 5-hydroxytryptophan (5HTP)
O = 5-hydroxyindoleacetic acid (5HIAA)
* = N-acetylserotonin
X = serotonin
Δ = N_{ω}-methylserotonin (internal standard).

b) PPP. The blood was centrifuged immediately at 4°C for 1 h at 1,720 x g, and to avoid inclusion of platelets trapped in the floating lipid layer or from the plasma/red cell interface, the middle third of the supernatant (PPP) was removed and stored frozen at -20°C.

For normal male subjects (n=12), mean PRP platelet count (± SD) was 480.3 ± 150.2 x 10^9/l (Table 3.1). For PRP prepared from the same individual (male) over a period of several months, the platelet counts were reproducible (mean ± SD, 437.9 ± 55.5 x 10^9/l, n=8). Mean percentage contamination by white blood cells and red blood cells was 0.1% and 6.2% for a given individual, and 0.2% and 6.2% between volunteers respectively (calculated as a percentage of the mean number of platelets). The corresponding values for white and red cell contamination calculated as a percentage of the mean normal whole blood ranges were 6.7% and 0.5% for a given individual, and 10.7% and 0.5% between volunteers respectively.

3.2.6 Preparation of Rat Serum for HPLC-ECD Serotonin Assay

Two methods were used to prepare rat serum:

a) Neat serum.

Whole blood (1-5 ml) was obtained from ether-anaesthetised rats by cardiac puncture using a 19 gauge needle and dispensed into a plain polystyrene tube (10 ml). The blood was allowed to clot at 4°C for 2-4 h, before being centrifuged at 1,720 x g for 1 h at 4°C, the supernatant collected, and frozen at -20°C.

PRP Platelet Parameter	Intra-Individual Value (n=8) Mean ± SD	Inter-Individual Value (n=12) Mean ± SD	Normal Whole Blood Ranges
Platelet Count ($\times 10^9/l$)	437.9 ±55.5	480.3 ±150.2	150-400
MPV (fl)	9.3 ±0.3	9.2 ±1.0	6-10
WBC ($\times 10^9/l$)	0.5 ±0.2	0.8 ±0.3	4-11
RBC ($\times 10^{12}/l$)	0.03 ±0.005	0.03 ±0.007	5-7

Table 3.1 PRP parameters compared with normal whole blood values in normal males.

MPV = mean platelet volume

WBC = white blood cell count

RBC = red blood cell count

b) Diluted serum.

Whole blood (100 µl) was either collected as above, or via an indwelling arterial cannula from conscious rats. The blood was added to a saline solution (900 µl) which contained thrombin (1 unit), chlorimipramine (1 nmol), and pargyline (10 nmol). The diluted sample was mixed by inversion, and left to clot for 2-4 hr at 4°C, before centrifugation for 30 min at 20,000 x g and 4°C. The supernatant was frozen at -20°C prior to assay.

3.2.7 Acid Deproteinisation of PRP/Serum Samples for HPLC-ECD Assay

3.2.7.1 Purification of [^{3}H]-Serotonin for Recovery

Although the tritiated tracer as received from Amersham was usually >95% pure, once the vial had been opened, the purity (as measured by HPLC-ECD analysis) rapidly decreased over a period of weeks (Figure 3.6). Thus it was necessary to re-purify the tracer by use of Amberlite ion-exchange resin. Amberlite CG-50 (0.15 g) was prepared by packing as a slurry (in 0.1 mol/l hydrochloric acid) into a 1 ml polypropylene syringe, the exit being blocked by a glass wool plug. The column was washed with 0.1 mol/l phosphate:1 mmol/l EDTA buffer (10-15 ml), pH 7, until the eluate was neutral, followed by distilled water (15 ml) until the pH of the eluate was constant (pH 5-6). Tritiated serotonin (10 µl; 10 µCi) was diluted in 0.1 mol/l pH 5 sodium acetate buffer (2 ml), applied to the column, and allowed to drain through; this was followed by a water wash (10 ml). The eluting buffer (0.2 mol/l sodium acetate, pH 4) was preceded by an intermediate wash of 0.1 mol/l pH 4.5 sodium acetate buffer

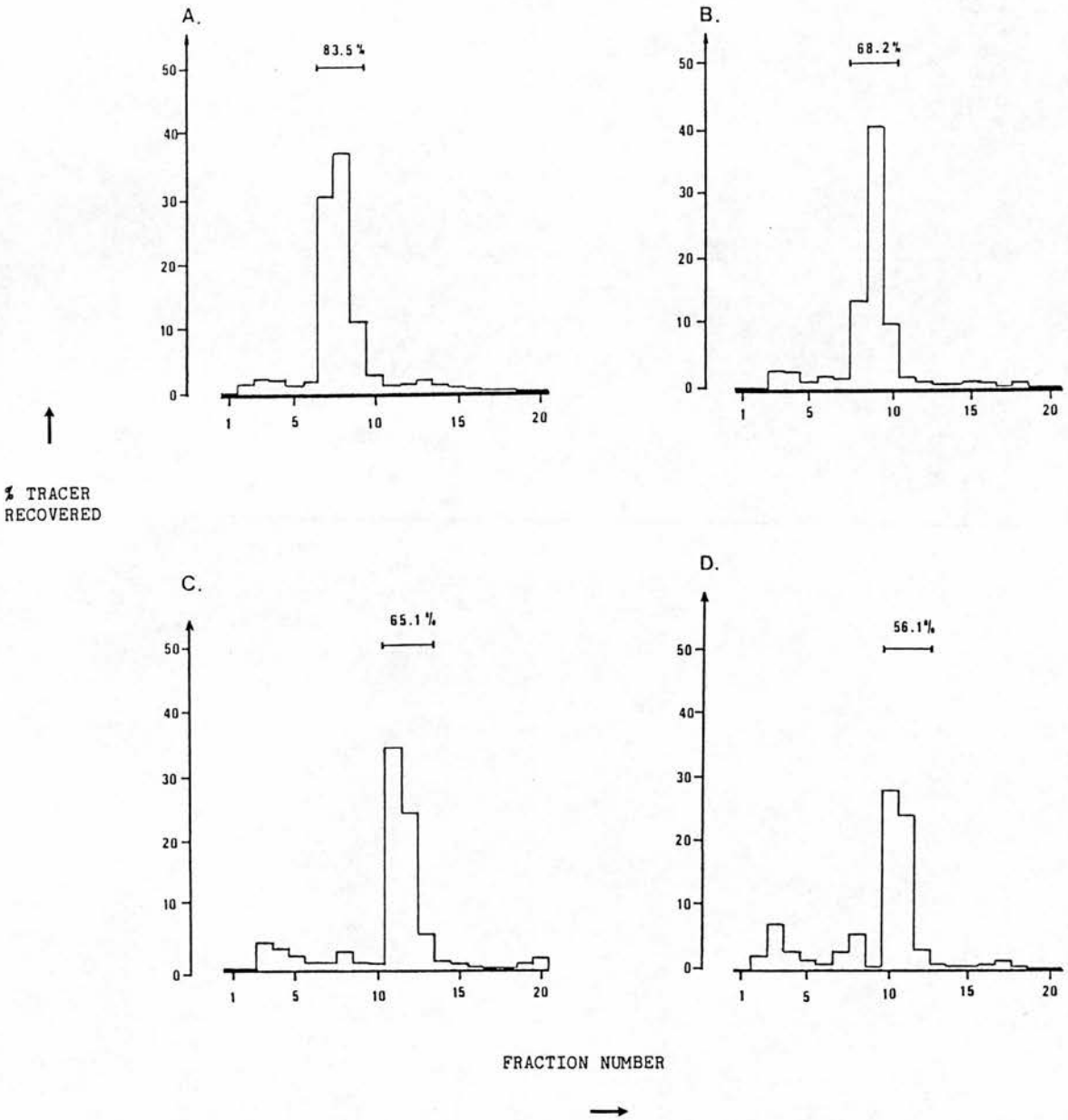


Figure 3.6 Decrease in purity of [^{3}H]-serotonin tracer as determined by HPLC analysis at intervals of a) 4 , b) 8, c) 12, and d) 16 weeks after opening the sealed vial. Value is % injected radioactivity recovered in the serotonin peak.

(10 ml). The eluate was collected in fractions (1 ml) and aliquots (100 µl) used for scintillation counting, and HPLC-ECD analysis.

An elution profile of tritiated serotonin from the Amberlite is shown in Figure 3.7. Analysis of fractions 27-28 by HPLC-ECD from four experiments showed that the tracer was 94 ± 1.6% pure (mean ± SD; n = 4) immediately after purification.

3.2.7.2 Recovery of [^{3}H] Serotonin From Plasma After Acid Deproteinisation as an Internal Standard for Serotonin

Freshly purified [^{3}H]-serotonin in assay buffer (50 µl; 30,000 dpm) was added to PRP (400 µl), vortexed, and left to equilibrate (15 min at room temperature) before being deproteinised: an aliquot (50 µl) of perchloric acid/cysteine (1.5 mol/l and 2.0 mmol/l respectively) was added to the sample (450 µl) in a conical polypropylene microcentrifuge tube (1.5 ml). The tube was vortexed, left for 15 min at 4°C, and then centrifuged at 10,000 x g for 15 min at 4°C. Duplicate aliquots (2 x 50 µl) of the protein-free supernatant were then removed for liquid scintillation counting. Totals consisted of aliquots (50 µl) taken from plasma which had been deproteinised and then spiked with purified tracer at the same dilution as for the recovery samples. Backgrounds were deproteinised platelet-poor plasma (50 µl) only.

Recovery of purified [^{3}H]-serotonin after deproteinisation from PRP was (mean ± SD) 92.0 ± 2.9% for samples of the same plasma (n=7), and 91.5 ± 1.8% for plasma from different normal volunteers (n=7). There was no significant difference between these two values as calculated by the Student's *t* test.

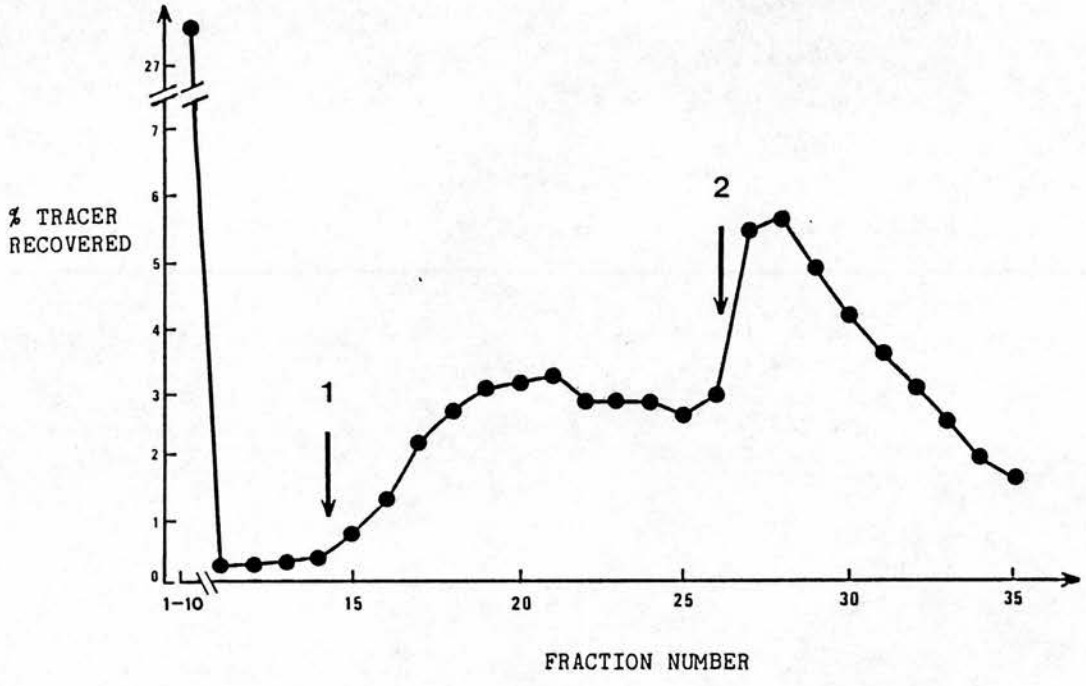


Figure 3.7 Elution profile of [^{3}H]-serotonin from the Amberlite column.

1 = 0.1 mol/l acetate, pH 4.5

2 = 0.2 mol/l acetate, pH 4.0

In order to see if significant degradation of serotonin would occur in human plasma, a sample of PRP (400 µl) was spiked with [^{3}H]-serotonin, left to equilibrate at room temperature for 15 min, then incubated for 1 h at 37°C. The sample was then frozen and thawed to disrupt the platelets, and analysed by HPLC-ECD. The elution profile (Figure 3.8) showed that 99.7 % of the original [^{3}H]-serotonin (tracer co-injected with deproteinised PRP) was still present. No distinct peak corresponding to 5HIAA was seen. Thus it was concluded that no appreciable degradation of PRP serotonin should occur during normal sample processing, which is in good agreement with previous work (Born and Gillson, 1959).

3.2.7.3 Recovery of Nω-Methylserotonin from Human Plasma and Rat Serum After Acid Deproteinisation as an Internal Standard for Serotonin

Human PRP or rat serum (400 µl) was spiked with 1.25 nmol Nω-methylserotonin diluted in saline (50 µl) and was deproteinised as described above. Aliquots (25 µl) of supernatant were assayed for recovery by HPLC, which had previously been calibrated with Nω-methylserotonin. This internal standard was used for all subsequent analytical runs with the HPLC-ECD.

Recovery of Nω-methylserotonin from samples of the same human plasma was (mean ± SD) 80.4 ± 3.7% (n=7), and from different plasmas, 83.3 ± 7.9% (n=31). There was no significant difference between these two figures (Student's *t* test), but they were both significantly lower than the corresponding recoveries of tritiated

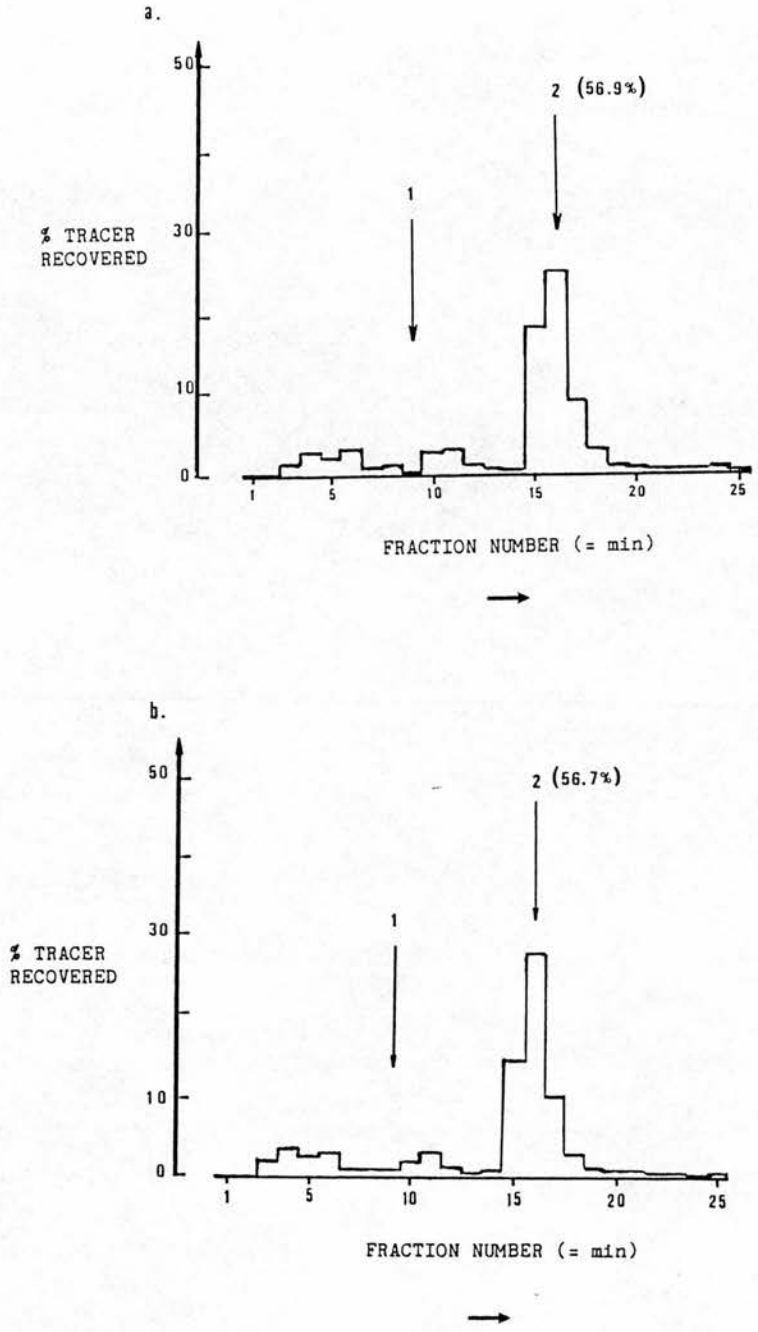


Figure 3.8 Effect of incubating human PRP at 37°C on the elution profile of [^{3}H]-serotonin from the HPLC-ECD:

a) PRP incubated for 1h at 37°C, freeze-thawed, then deproteinised and co-injected with tracer

b) PRP spiked with [^{3}H]-serotonin, incubated for 1 h at 37°C, freeze-thawed, then deproteinised and injected

1 = position of 5HIAA peak, 2 = position of serotonin peak.

Figure in brackets = % of injected radioactivity recovered in the serotonin peak.

serotonin ($p<0.001$). Once deproteinised and stored at 4°C, the serotonin in the PRP samples was stable for at least 6 h (Table 3.2).

A lower mean recovery of internal standard was seen with neat rat serum when prepared directly from cardiac puncture (mean ± SD, 71.3 ± 18.0%, n=84); also, many of the samples were distinctly haemolysed. The mean recovery of HPLC-ECD internal standard from rat serum prepared from rat blood collected into the "coagulant" mixture was (mean ± SD) 89.1 ± 19.0% (n=73). This was significantly higher than the recovery from neat serum ($p<0.005$, Student's t test). Serotonin was acceptably stable in these samples: diluted serum incubated in triplicate at 37°C for up to two hours prior to HPLC-ECD analysis showed no significant decrease in serotonin concentration (Table 3.3).

3.2.8 Oxidative Voltammograms of the Putative Serotonin Peak from Human PRP and Rat Serum

PRP was prepared and deproteinised, and aliquots (25 µl) analysed alternately with a standard solution (25 µl) of serotonin in acid/cysteine (1 µmol serotonin/l), the applied potential being decreased after each injection of standard, as described above (3.2.4), to allow the construction of oxidative voltammograms. In a separate experiment, rat serum was prepared and deproteinised, and alternate injections (25 µl) made with a standard serotonin solution in acid/cysteine (4 µmol serotonin/l) as described above for human PRP.

The oxidative voltammograms for standard serotonin and human PRP are coincident (Figure 3.9), confirming that the PRP peak behaves

Time (min)	Serotonin (nmol/l)
0	1338.1
40	1323.9
71	1352.1
105	1348.0
259	1315.5
288	1323.9
303	1323.9
392	1406.7

Table 3.2 Stability of serotonin in deproteinised PRP at 4°C.

Time at 37°C (min)	Serotonin Concentration (mean ± SD, nmol/l)	p Value (Student's t Test)
0	14,066 ± 596	--
30	13,804 ± 523	NS
60	14,980 ± 232	NS
90	12,982 ± 1381	NS
120	13,326 ± 1538	NS

Table 3.3 Effect of incubating diluted rat serum at 37°C.

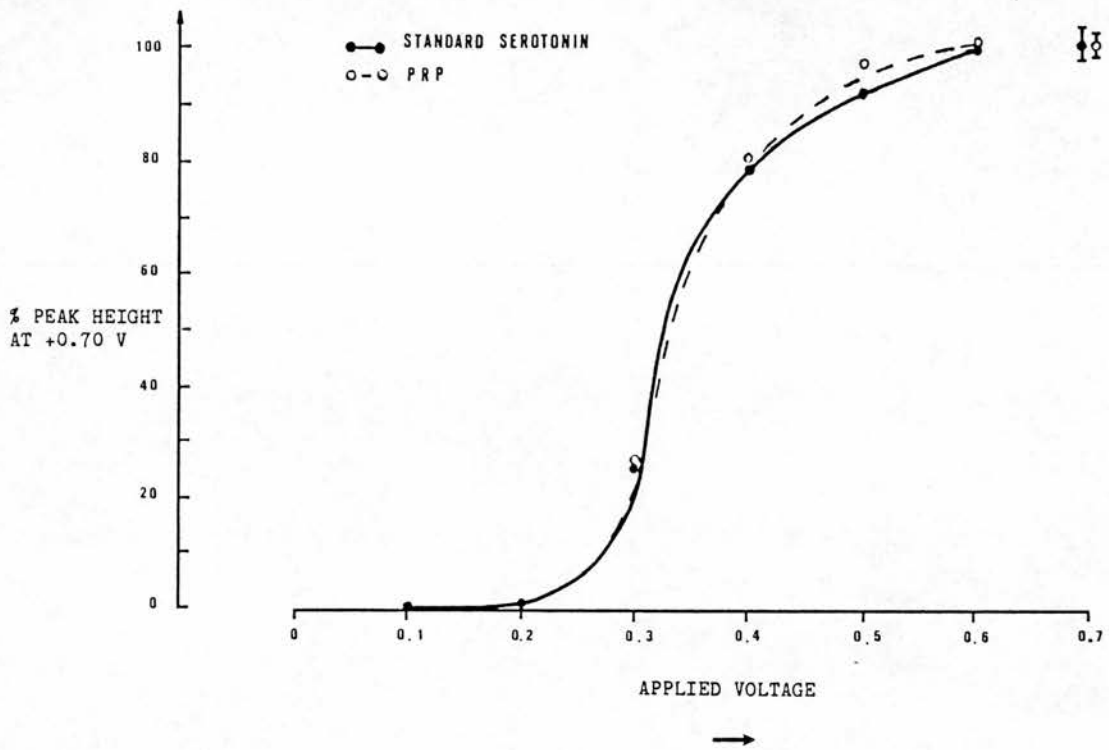


Figure 3.9 Oxidative voltammogram for standard serotonin and human PRP serotonin.

identically to authentic serotonin under the same conditions for oxidation. The retention times (mean ± SD) of the standard serotonin peak, and the putative PRP serotonin peak were 8.95 ± 0.01 min and 8.86 ± 0.31 min respectively (n=6). These values were not significantly different when compared by Student's t test. On co-injection of standard serotonin with human PRP, the height of the resulting peak was 101.2% of the arithmetic sum of the two individual peak heights.

The oxidative voltammograms constructed from the rat serum and standard serotonin injections were superimposable (Figure 3.10). The retention times (mean ± SD) for six consecutive injections each of deproteinised rat serum and standard serotonin were not significantly different (10.82 ± 0.12 min, and 10.87 ± 0.27 min respectively, Student's t test). Furthermore, co-injection of a standard serotonin solution and a deproteinised rat serum sample gave a peak which was 97.5% of the arithmetic sum of the two individual peak heights.

3.3 Development of a Radioimmunoassay for Serotonin

3.3.1 Synthesis of N-Succinamylserotonin and Immunogen

N-Succinamylserotonin was synthesised and conjugated to bovine serum albumin by Dr. John Corrie, (MRC Clinical and Population Cytogenetics Unit, Western General Hospital) using methods modified from those of Delaage and Puizillout (1981): serotonin creatinine sulphate (61 mg) and succinic anhydride (91 mg) were stirred under nitrogen for 10 min with 0.68 mol/l potassium hydroxide (2.16 ml).

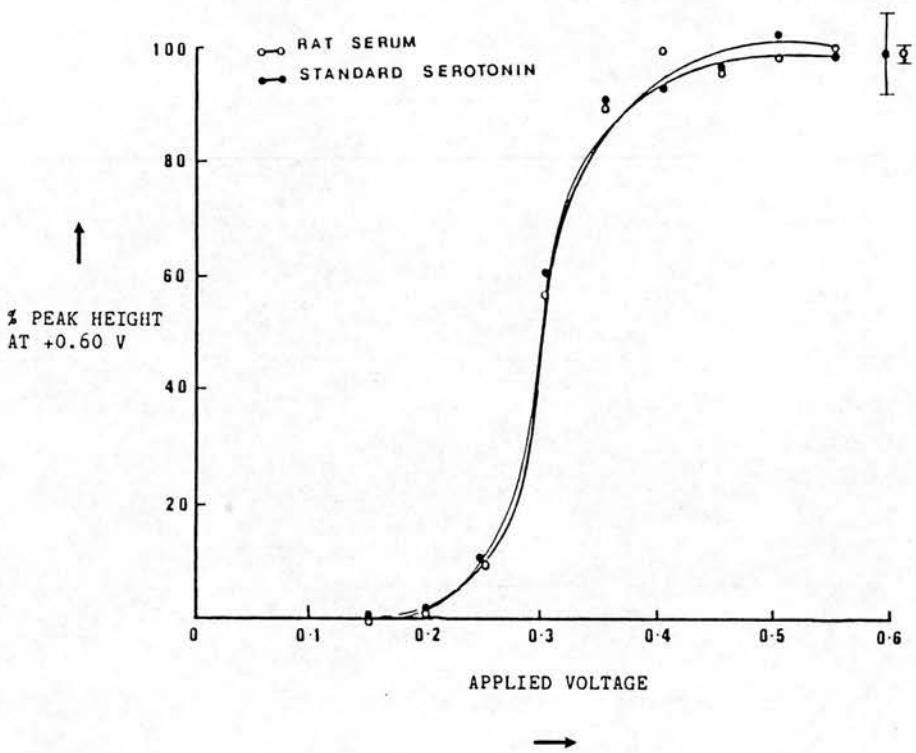


Figure 3.10 Oxidative voltammogram for standard serotonin and rat serum serotonin.

A solution of hydroxylamine hydrochloride (21 mg) in water (0.5 ml) was added, followed by 0.68 mol/l potassium hydroxide (1.55 ml), and the solution was stirred for a further 5 min, then adjusted to pH 4 by addition of 1 mol/l hydrochloric acid. The solution was rapidly applied to a column of Amberlite XAD-2 (25 ml; previously freed of UV-absorbing contaminants by exhaustive washing with ethanol) in water, and the column was then washed with water (approximately 200 ml) until the eluate showed no UV absorption. The column was eluted with ethanol (150 ml), and the yield of N-succinamylserotonin determined by its UV absorption at 278 nm (ξ_{278} = 5,740 l/mol.cm).

The ethanolic solution was evaporated under reduced pressure, the residue redissolved in water (10 ml), and lyophilised to leave the product as a pale foam, which was either used directly for conjugation to bovine serum albumin, or redissolved in dry dimethylformamide (DMF) at 2.8 mg/ml. Aliquots of this solution were sealed under nitrogen in glass ampoules to avoid oxidation and stored at -20°C for use in the preparation of the radioiodinated tracer.

For the preparation of the immunogen, a solution of N-succinamylserotonin (38 mg) in dry dioxan (2.14 ml) was cooled to 10-12°C, and treated with tri-n-butylamine (62 µl), and isobutyl chloroformate (15 µl). After 20 min this solution was added under nitrogen to an ice-cold solution of bovine serum albumin (332 mg) in water (8.8 ml), 1 mol/l sodium hydroxide (0.33 ml) and dioxan (5.7 ml). This solution was stirred in an ice bath for 1 h, then treated with 0.25 mol/l sodium phosphate, pH 6 (1.4 ml), and dialysed for 1 h against 0.05 mol/l sodium phosphate, pH 6 (5 l). The dialysate was subjected to

gel filtration on Sephadex G-50 (40 x 3 cm), eluting with 0.05 mol/l sodium phosphate, pH 6. The fraction eluted in the void volume was analysed by UV spectroscopy and Lowry protein estimation, which showed a conjugation ratio of 24 serotonin residues per protein molecule. The eluate was lyophilised and the conjugate stored at -20°C.

3.3.2 Production of Antisera

Initially, six guinea pigs (Western General Hospital Animal Unit stock) were used, then later, four rabbits (New Zealand White): the guinea pigs were injected with conjugate at either 4 sites sub-cutaneously, or 1 site intra-peritoneally, and for the rabbits, immunisation was always at 4 sites sub-cutaneously. The dose was 100 µg/animal in each case, in 1:1 Freund's complete adjuvant:saline for the primary, and in 1:1 Freund's incomplete adjuvant:saline for the boosts. Boosts were given six weeks after the primary immunisation, and thereafter at intervals of six weeks; bleeds were taken ten days after boosting, the first bleed being taken after the first boost. Blood was collected as heparinised plasma for the early bleeds, centrifuged at 1,720 x g for 60 minutes at 4°C, and the platelet-poor plasma harvested; serum was prepared from later bleeds. The plasma or serum was stored neat, or in a 1/10 dilution in assay buffer (0.1 mol/l citric acid, 0.3 mol/l sodium hydroxide, 1 mmol/l EDTA, 0.1% gelatine, final pH 6.2) at -20°C.

3.3.3 Synthesis of Radioactive Tracers

3.3.3.1 Synthesis of a Succinylated [^{3}H]-Serotonin Tracer

Tritiated serotonin (10 µl; 10 µCi) was diluted from an Amersham stock into pH 7.4, 0.05 mol/l phosphate buffer (10 ml). An aliquot of this (1 ml) was added to succinic anhydride (5 mg) in a small glass tube, vortexed to dissolve the anhydride, a volume of triethylamine added (20 µl), and the tube vortexed again. The tube was then incubated at 4°C overnight, and the putative tracer purified by injecting a sample (750 µl) onto the HPLC-ECD (previously calibrated with authentic N-succinamylserotonin), and the fractions corresponding to the N-succinamylserotonin peak collected (approximately 2 ml). The tracer was diluted before use in assay buffer (0.1 mol/l citric acid, 0.3 mol/l sodium hydroxide, 1 mmol/l EDTA, 0.1% gelatine, final pH 6.2).

A profile of tritiated material eluting from the HPLC-ECD is shown before, and after succinylation (Figure 3.11). This tracer was used for studying immunoreactivity of the early bleeds, prior to synthesis of a gamma-emitting tracer.

3.3.3.2 Synthesis of a Gamma Emitting Tracer: Using N-Succinamyl-serotonin Conjugated to [^{125}I]-Iodinated Glycyl Tyrosine

The procedure used was similar to that already described (Delaage and Puizillout, 1981). All solutions were freshly prepared on the day of the iodination. An aliquot (50 µl) of N-succinamyl serotonin in DMF (2.8 mg/ml) was taken from one of the sealed vials,

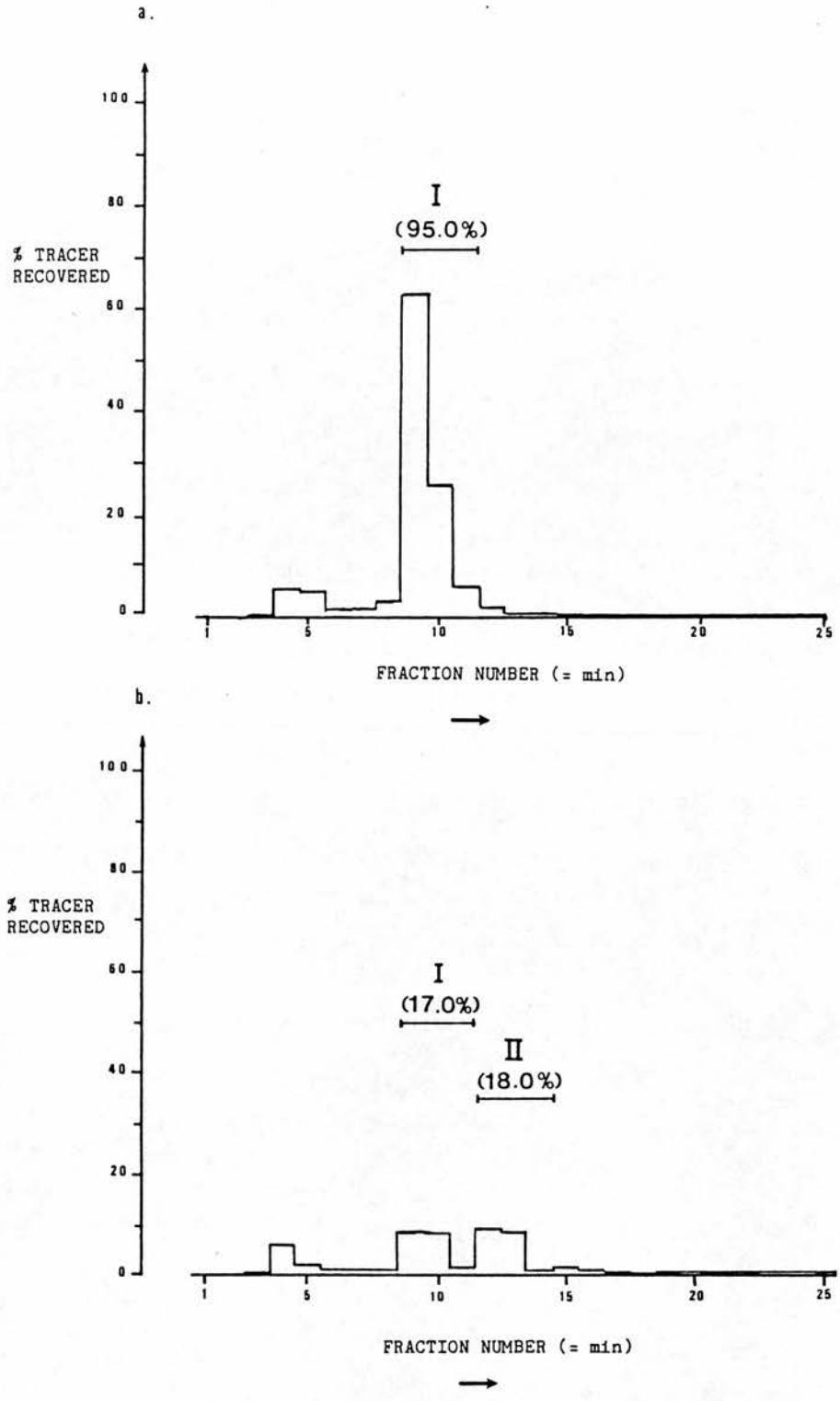


Figure 3.11 Elution profile of [^{3}H]-serotonin from the HPLC-ECD system a) before and b) after succinylation. Figure in brackets = % injected radioactivity recovered in peak.

I = serotonin

II = N-succinamylserotonin

and dispensed into a conical polypropylene microcentrifuge tube. For activation of the N-succinamylserotonin prior to conjugation, a solution (10 µl) of tri-n-butylamine in DMF (17 µl/ml) was added, followed by a solution (10 µl) of isobutyl chloroformate diluted in DMF (45 µl/8.5 ml). The tube was vortexed and incubated for 20 min at 10-12°C. Glycyl tyrosine (476 ng) in 0.25 mol/l pH 7.5 phosphate buffer (10 µl) was added to a 1 mCi solution (10 µl) of Na[^{125}I] (specific activity 1,650 - 2,157 Ci/mmol) and mixed. Chloramine T (10 µg) dissolved in the same phosphate buffer (10 µl) was added to the [^{125}I]-iodine/glycyl tyrosine solution, mixed, and left at room temperature for 1 min. The reaction was stopped by the addition of sodium metabisulphite (12 µg) in 0.05 mol/l phosphate buffer, pH 7.5 (20 µl). An aliquot of activated N-succinamylserotonin solution (50 µl) was added to the [^{125}I]-iodinated glycyl tyrosine, the solutions mixed, and left at 4°C for 5 min. The reaction mixture was diluted with 500 µl of the eluting buffer (0.1 mol/l citric acid, 0.1 mol/l sodium chloride, 3 mmol/l ascorbic acid, final pH 6.2) before being applied to a Sephadex G-25 column (49 x 1 cm). The buffer was pumped through the column at 1 ml/min, and fractions (6 ml) collected in plain polystyrene tubes (10 ml). Aliquots (10 µl) of the fractions were counted in a multiwell gamma-counter to locate the peaks. The immunoreactive fractions of a single peak (typically fractions 25-30) were pooled, diluted 1+1 with assay buffer, and stored at -20°C as aliquots (2 ml) in stoppered polystyrene tubes. Each aliquot was thawed once only, after which it was stored in the dark at 4°C and discarded after 1 week.

The gamma-emitting material eluting from the Sephadex column after the application of the iodination mixture shows two distinct peaks (Figure 3.12). When an aliquot of pooled material from the later eluting peak was incubated with excess antiserum, >90% of the added tracer was recovered in the bound fraction. Analysis of pooled fractions from this peak showed that the specific activity was 2,011 Ci/mmol, and the yield of tracer was 28.3 $\pm$ 8.5% (mean $\pm$ SD, n=5). If the mass of tracer added was kept constant from assay to assay, the tracer was usable for up to five months after iodination, with no apparent loss in immunoreactivity. This method of iodination was used to prepare all subsequent gamma-emitting tracers for use in the studies described in this thesis.

3.3.3.3 Synthesis of a Gamma Emitting Tracer: Conjugation of Serotonin to N-Succinimidyl-[^{125}I]-(3-iodo-4-hydroxyphenyl)-propionate, ([^{125}I]-NSHPP, [^{125}I]-Bolton-Hunter Reagent)

The NSHPP ester was iodinated as previously described (Bolton and Hunter, 1972), and extracted with toluene (200 µl) by vortexing for 1 min. The organic layer was removed to a clean, glass centrifuge tube, and the iodination tube given four toluene washes (4 x 200 µl), which were also added to the centrifuge tube. The toluene was evaporated under vacuum, whilst gently warming the tube in a warm water-bath. The residue was washed down to the tube tip with toluene (200 µl), which was evaporated as before; this step was repeated once more before the addition of serotonin (10 µl of 900 µg/ml) in borate buffer (0.1 mol/l, pH 8.5). The mixture was

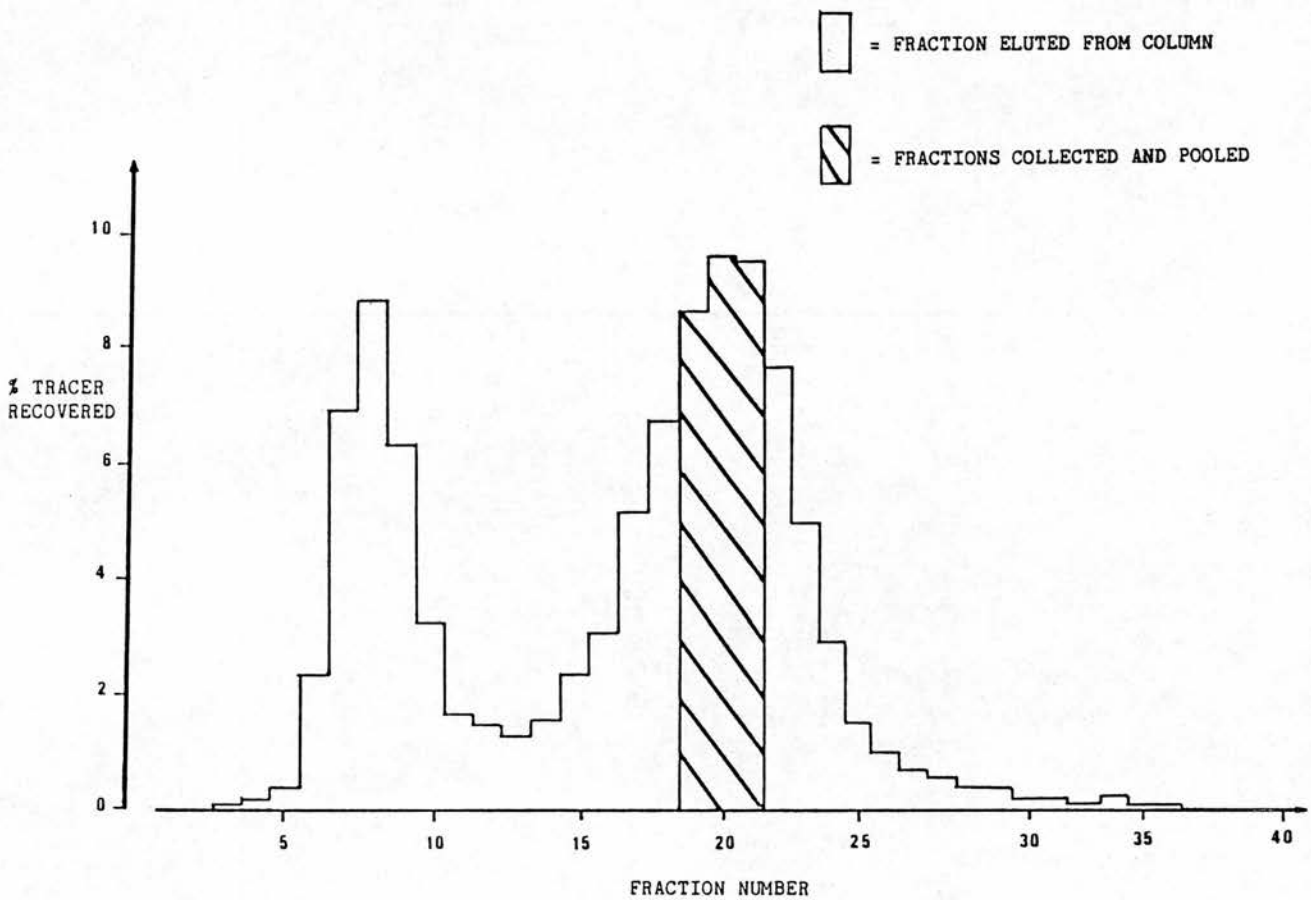


Figure 3.12 Elution of gamma-emitting material from the Sephadex column during the purification of N-succinamylserotonin conjugated to [^{125}I]-iodinated glycyl tyrosine.

incubated at 0°C in an ice/water slurry for 30 min before the addition of glycine (300 µl of 0.1 mol/l), after which the tube was left a further 5 min. The putative tracer was extracted by adding ethyl acetate (300 µl) and vortexing for 1 min, after which the organic layer was removed, evaporated under reduced pressure, and reconstituted in a smaller volume of ethyl acetate (50 µl). This was spotted onto a silica TLC plate (200 x 200 x 0.25 mm), and developed in ethyl acetate:methanol (9:1). The plate was allowed to dry in air, and the peaks located after a 30 min exposure to X-ray film. The major peak was scraped off the plate into ethanol (5 ml) in a glass tube (10 ml). After vortexing for 30 s and centrifuging down the silica, an aliquot was taken for testing in an antibody dilution curve, and the remainder stored at -20°C.

Three bands of radioactivity were obtained (Figure 3.13), with R_f 0.09, 0.11, and 0.36. Only the major peak (R_f 0.36) was tested, but less than 15% of added activity was bound in the presence of excess antibody, previously shown to bind approximately 90% of tritiated tracer. This tracer was not studied further.

3.3.4 Testing of Antisera

Varying dilutions of immune plasma/serum (or non-immune plasma or serum for non-specific binding, NSB) were made in assay buffer, and added (100 µl) to tubes containing buffer (200 µl) and a fixed dilution of tracer (100 µl). The tubes were vortexed, and incubated overnight at 4°C. Separation was by one of the following methods:

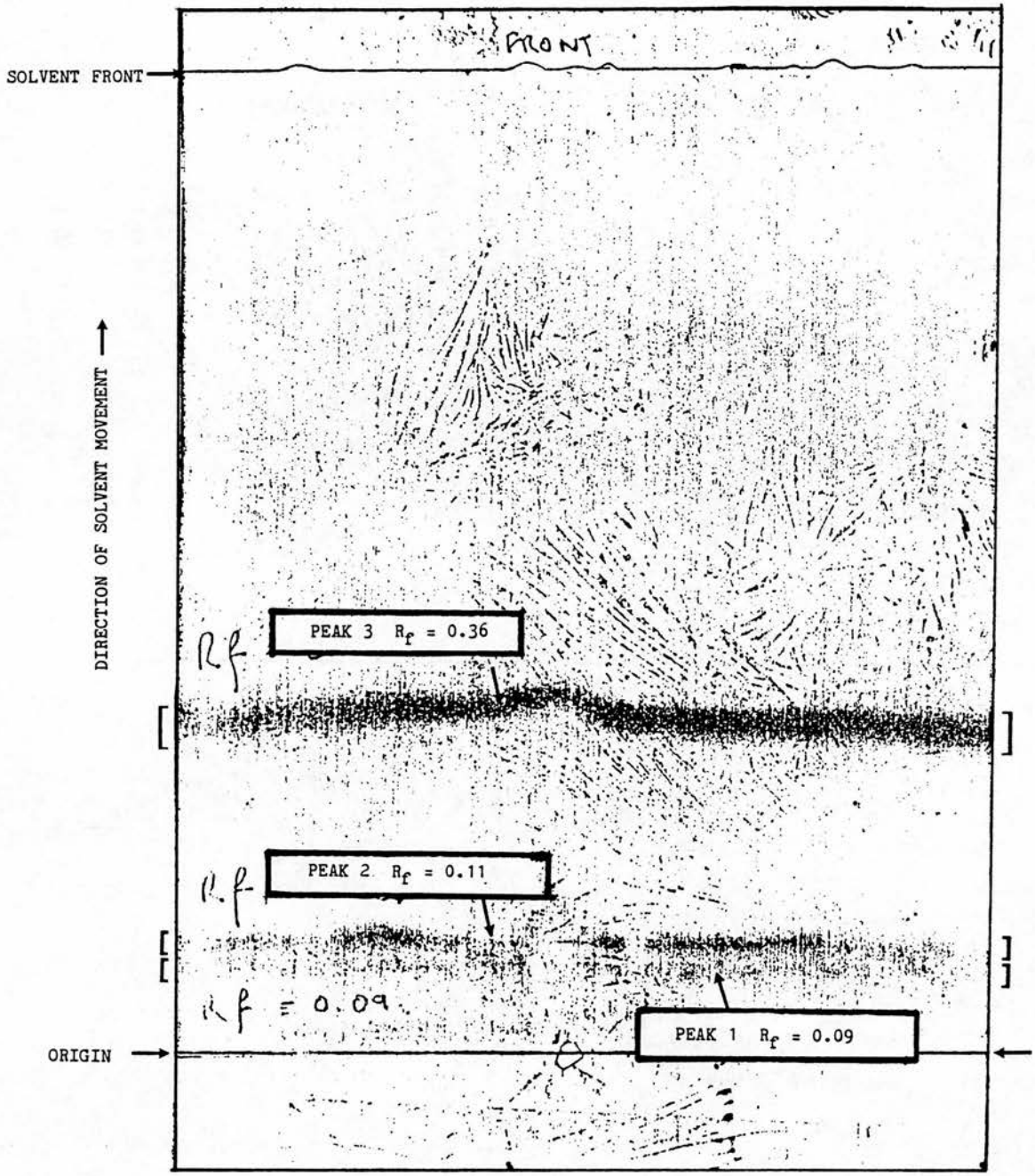


Figure 3.13 Distribution of radioactivity on the TLC plate after the purification of serotonin-[^{125}I]-NHSPP.

a) Ammonium Sulphate Precipitation

To each incubate (400 µl) was added a cold (4°C), saturated solution of ammonium sulphate (400 µl), the tube vortexed, and left at 4°C for 1 h. The tubes were centrifuged at 1,720 x g for 1 h at 4°C, the supernatant removed by aspiration, the pellet washed with saturated ammonium sulphate (400 µl), then centrifuged for a further 1 h under the same conditions. The supernatant was aspirated, the pellet redissolved in water (1 ml) and then decanted into polypropylene scintillation vials, followed by scintillant (10 ml). Each vial was vortexed (1 min) and counted. This separation system was used only for the tritiated tracer.

b) Dextran Coated Charcoal (DCC)

DCC was prepared by suspending charcoal powder (1g) in a solution of dextran T-70 (0.1g) in assay buffer (1 l). The stirred charcoal solution (1 ml) was added cold (4°C) to each incubation tube (400 µl), and the tubes centrifuged immediately at 1,720 x g for 20 min at 4°C. For tritiated tracers, the supernatants (bound fraction) were decanted directly into scintillation vials for counting, and for the [^{125}I] tracers, the supernatant was aspirated, and the charcoal pellet (free fraction) counted. This system was only used to monitor activity of early bleeds.

c) Second Antibody Precipitation

The ratio of first antibody:second antibody:non-immune serum was optimised for both guinea pigs and rabbits as follows: incubates were set up as normal for three concentrations of first antibody

(approximately 1/1,000, 1/5,000, 1/15,000 final dilution in primary incubate) and incubated for 4 h at room temperature. This was followed by the addition of non-immune serum (100 µl) in the range 1/50-1/1,600 initial dilution in assay buffer, and then by the second antibody solution (100 µl) in the range 1/5-1/160 initial dilution in buffer; the tubes were vortexed, and incubated overnight at 4°C. An assay buffer prewash (1 ml) was added cold (4°C), and the tubes centrifuged at 1,720 x g for 20 minutes at 4°C. The supernatants were aspirated and the pellets (bound fraction) were counted in a multiwell gamma-counter.

All animals which were immunised gave sera or plasma which bound tracer, and could be displaced by authentic N-acetylserotonin. No difference was seen when blood was collected as either heparinised PPP, or as serum (Figure 3.14). A typical antiserum dilution curve is shown using either the tritiated tracer, or the gamma-emitting tracer (Figure 3.15). Approximately 80-90% of the tritiated N-succinamylserotonin could be bound by an excess of antiserum, and non-specific binding (NSB) was less than 5%. Using the gamma-emitting tracer, specific binding was >90%, NSB was <5%.

The rise in titre over the period of immunisation is shown for one of the rabbits, No. R16 (Figure 3.16). Results from an optimisation of second antibody (Table 3.4) show that with these batches of reagents, optimal precipitation of first antibody (1/28,000 final dilution) was achieved using final dilutions of 1/240 for the second antibody, and 1/4,800 for the non-immune carrier serum.

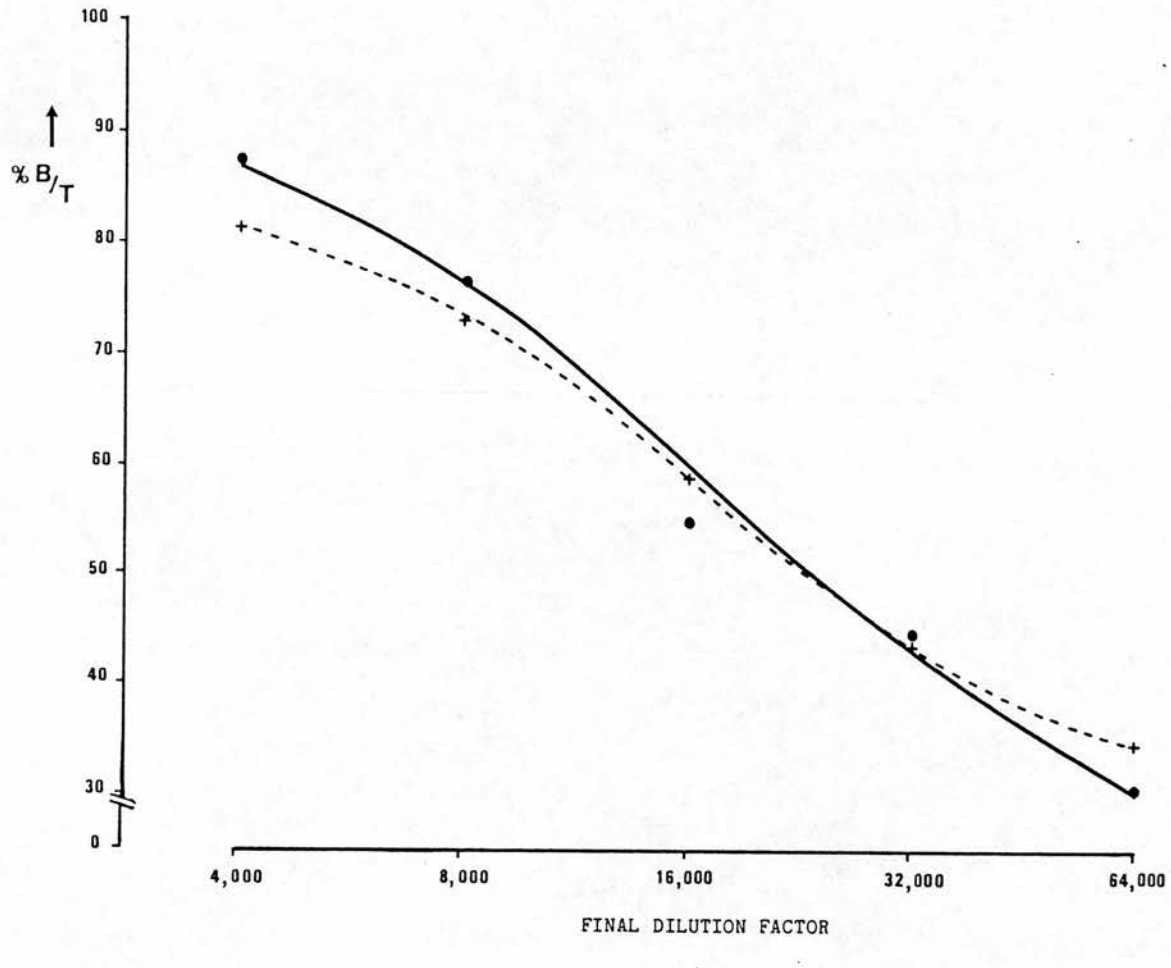


Figure 3.14 Antibody-dilution curve constructed using either heparinised plasma (●——●), or serum (+- - -+) prepared simultaneously from a rabbit immunised with the N-succinamylserotonin-BSA conjugate. Points represent means of duplicate estimations.

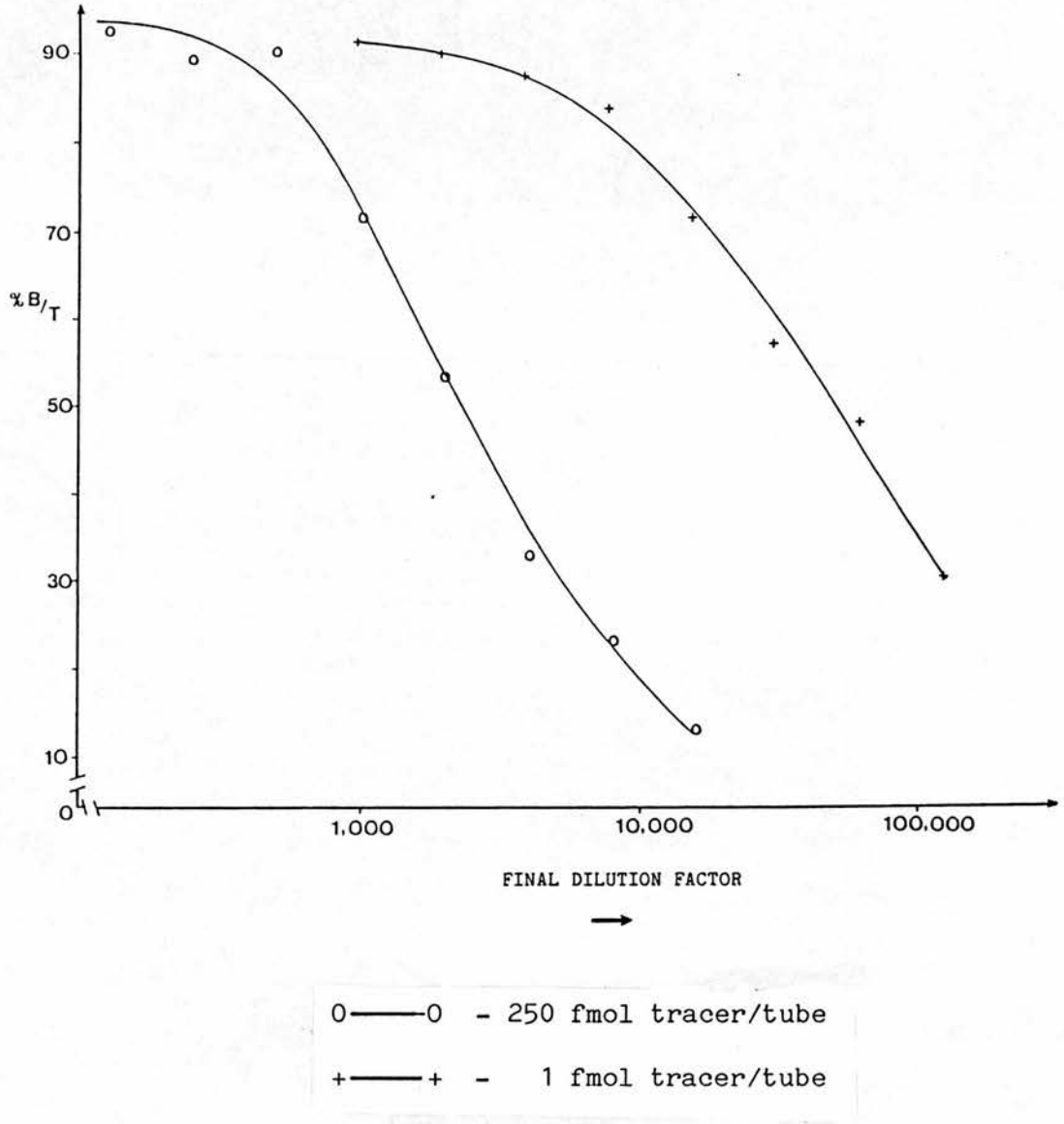


Figure 3.15 **Antibody dilution curves constructed using the same bleed of guinea pig plasma with either [^{3}H] (0——0) or [^{125}I] (+——+) as the radioactive molecule. Points represent means of duplicate estimations.**

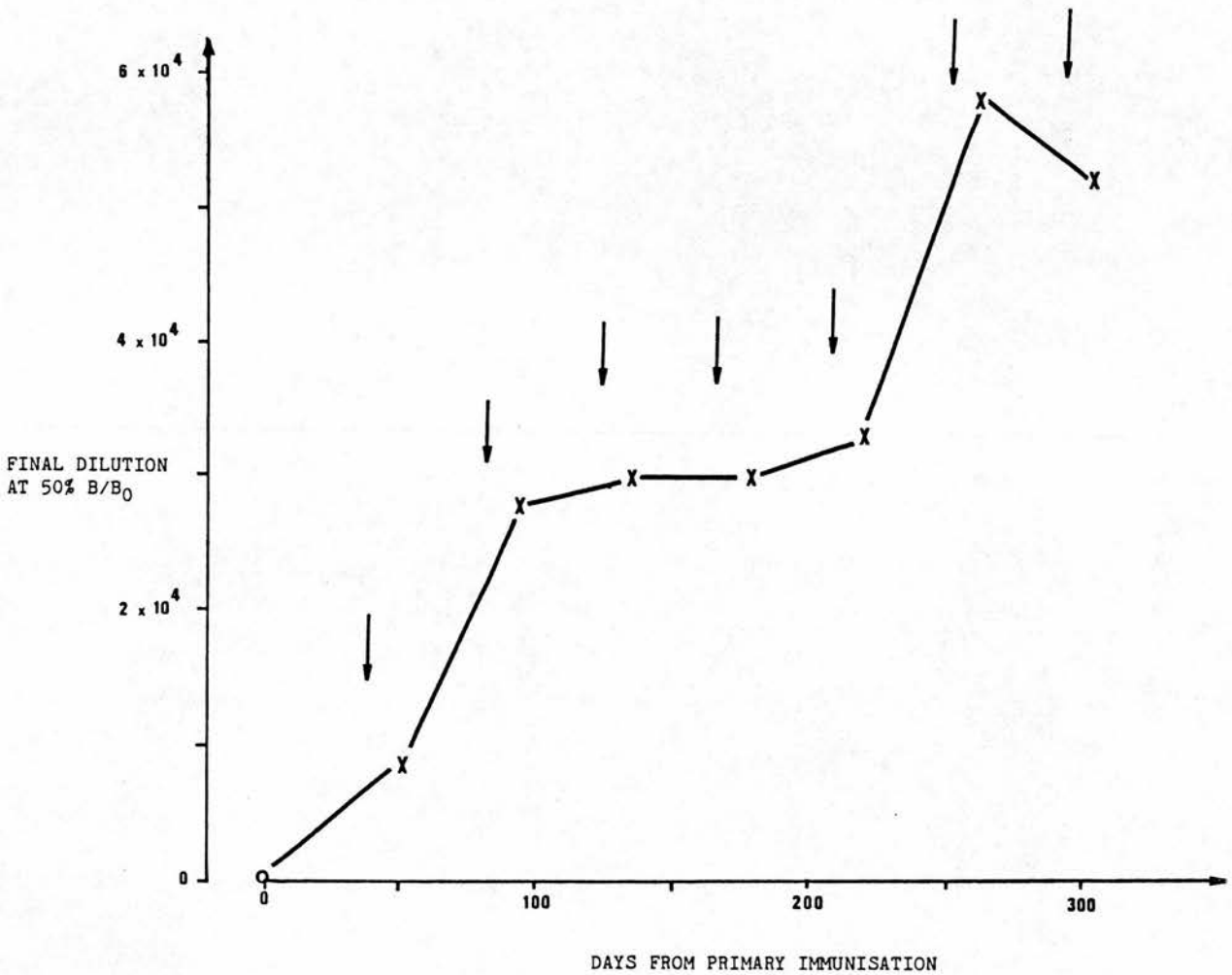


Figure 3.16 The rise in antibody titre in rabbit No. 16:

O = primary immunisation

↓ = boost

X = bleed

		NIRS final dilution factor					
		300	600	1,200	2,400	4,800	9,600
DARS final dilution factor	30	28.5	30.8	30.5	28.5	24.0	19.6
	60	32.1	37.5	37.7	37.4	35.1	28.6
	120	4.7	39.4	42.8	44.6	42.0	33.5
	240	3.8	6.1	42.8	46.8	45.1	41.7
	480	3.3	4.6	5.6	44.2	45.7	42.4
	960	3.6	4.9	5.2	6.1	40.3	41.5

Table 3.4 Typical second-antibody optimisation assay (% total tracer added recovered in the bound fraction)

NIRS = Non-immune rabbit serum

DARS = donkey anti-rabbit serum.

3.3.5 Standards and Cross-reactants

Standard serotonin, N-acetylserotonin, and putative cross-reactants were prepared in acid/cysteine as for the HPLC, and stored in the dark at 4°C. Concentrations were checked by UV absorption. Standards for the preliminary RIA standard curves were prepared fresh from N-acetylserotonin stock solution for each assay, in the range 1-1,000 nmol/l. A standard curve was set up by incubating the standards (200 µl) with tracer (100 µl), and first antibody solution (100 µl) at a dilution which was expected to give a zero standard binding (B_0) of approximately 50%.

Figure 3.17 shows standard curves using authentic N-acetyl-serotonin with guinea pig and rabbit antisera. One guinea pig serum (animal 17, bleed 6, GP17B6) and finally one rabbit serum (animal 16, bleed 4, R16B4) were selected on the basis of an acceptable titre coupled with good sensitivity. Both animals gave similar curves, with 50% B/B_0 being approximately 30 nmol/l, but the rabbit antiserum was usable at a higher dilution than the guinea pig serum.

3.3.5.1 Effect of Tracer Mass on the Standard Curve

Standard curves using N-acetylserotonin with guinea pig antiserum (GP17B6) were incubated with increasing masses of N-succinamylserotonin-[^{125}I]-glycyl tyrosine (approximately 0.6, 3.6, and 6.0 fmol/tube). Incubation and separation was by second antibody as above.

Increasing the mass of tracer added to the incubates did not result in a discernible shift or distortion of the standard curves

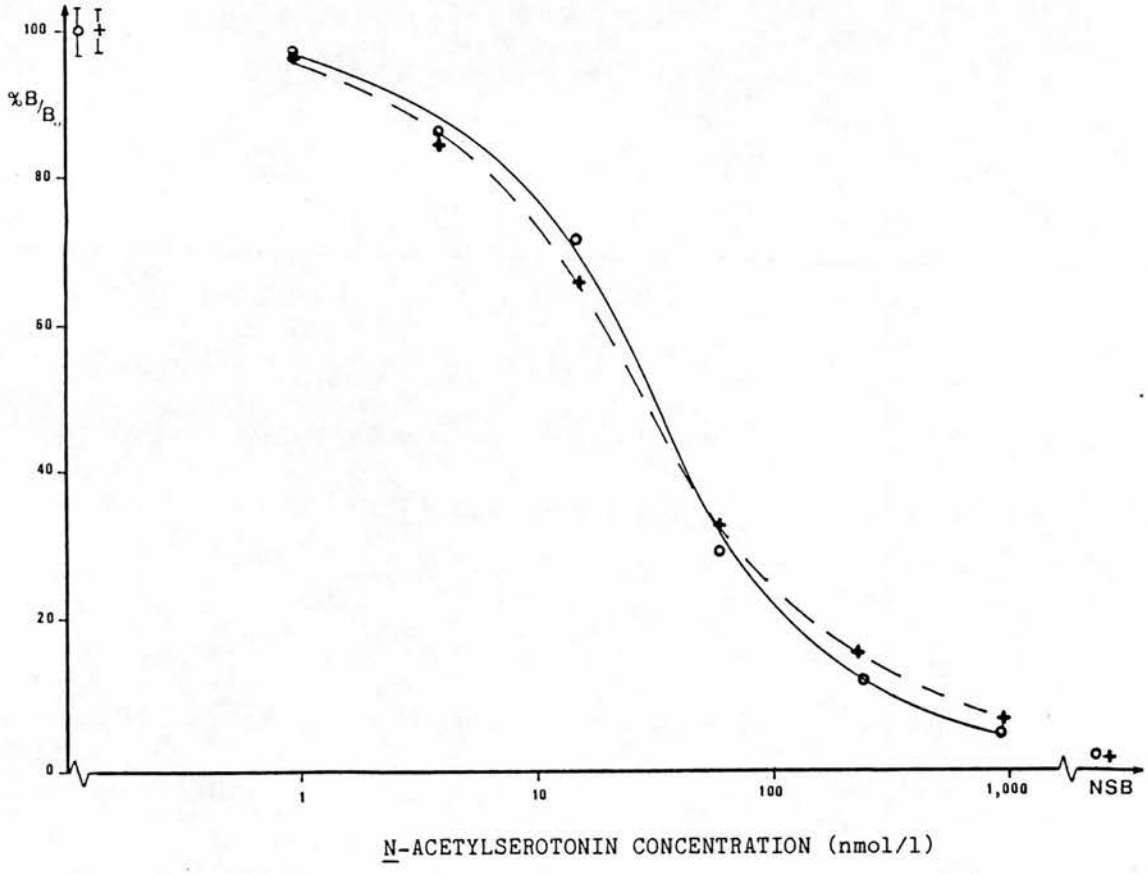


Figure 3.17 Standard curves constructed using standard N-acetylserotonin and either guinea pig (O——O; final dilution 1/10,500) or rabbit (+- - -+; final dilution 1/28,000) antisera. Points represent means of duplicate estimations; B_0 = mean ± SD of triplicates.

when plotted relative to the zero binding, B_0, (Figure 3.18), although a drop in B_0 was observed.

3.3.5.2 The Effect of pH on the Standard Curve

The effect of pH on standard curves with the guinea pig and rabbit sera was investigated by diluting all reagents in citrate buffers of differing pH values (4, 5, and 6.2). Incubation was as above, and separation was by second antibody. Lowering the pH markedly inhibited binding of tracer with the guinea pig antiserum from 60% tracer bound at pH 6.2, to 6% at pH 4.0 (Figure 3.19a). With the rabbit serum, there was a comparatively small drop, from 61% at pH 6.2, to 53% at pH 4 (Figure 3.19b). In view of this, the rabbit antiserum was used exclusively, so that any slight variations in pH would have a minimal effect on the standard curve.

3.3.5.3 Effect on the Standard Curve of Adding Antisera Separately, Pre-mixed, or as a Pre-precipitate

Standard curves were set up as described above using the rabbit antiserum, except that the primary antiserum, non-immune rabbit serum (NIRS), and donkey anti-rabbit serum (DARS), were added as:

a) individual aliquots of 100 µl each, after a 4 h pre-incubation at room temperature with the primary antiserum,
b) as a single aliquot of 300 µl, which was freshly mixed, or
c) as a single aliquot of 300 µl, from a resuspended mixture of the three sera (i.e. primary, NIRS, and DARS) which had been incubated overnight at 4°C to form a precipitate.

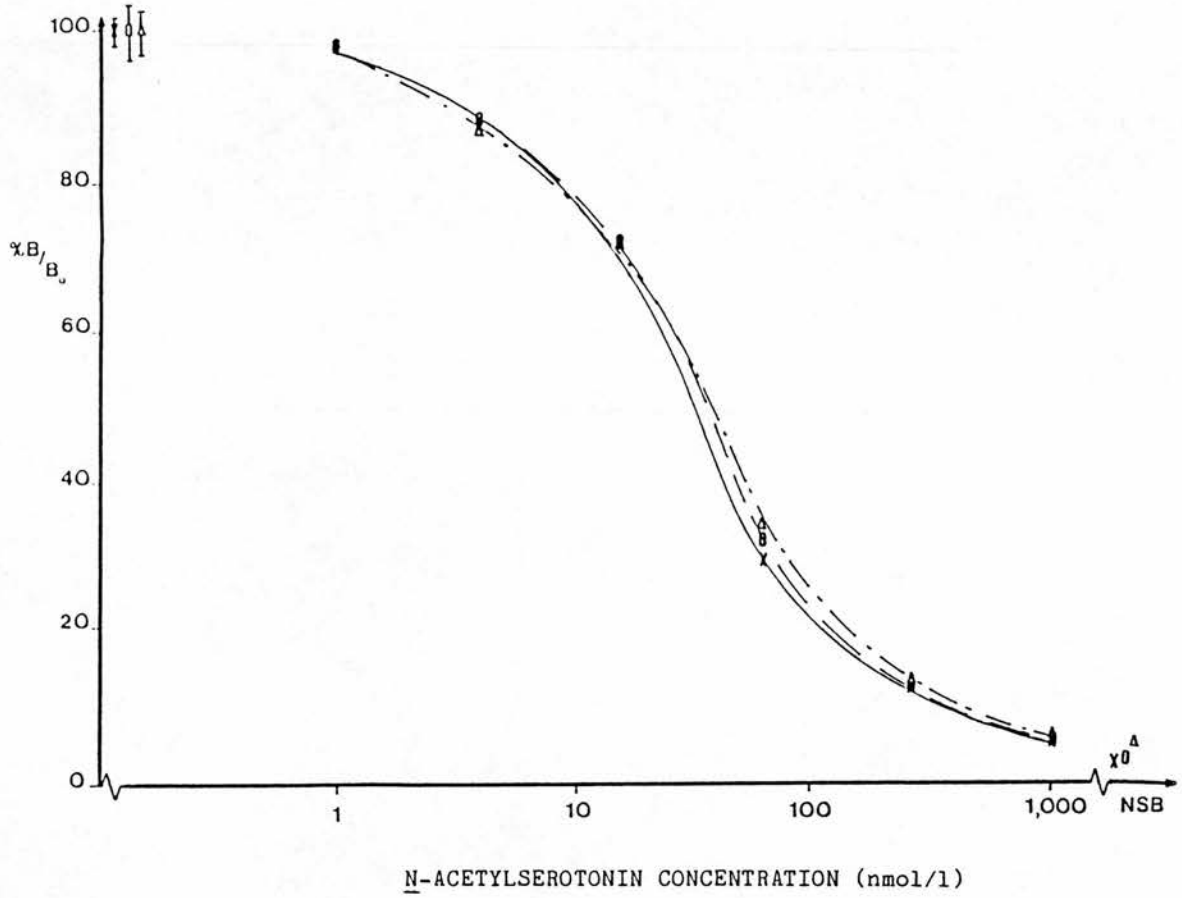


Figure 3.18 Effect of increasing tracer mass on the standard curve using guinea pig antiserum. Points represent means of duplicate estimations; B_0 = mean ± SD of triplicates.

X-----X = 0.6 fmol tracer/tube (B_0 = 64.9 ± 1.5%)

O- - -O = 3.6 " " " (B_0 = 54.7 ± 1.5%)

Δ- · -Δ = 6.0 " " " (B_0 = 43.9 ± 1.3%)

Hereafter, a tracer mass of 1 fmol/tube was used in the assay.

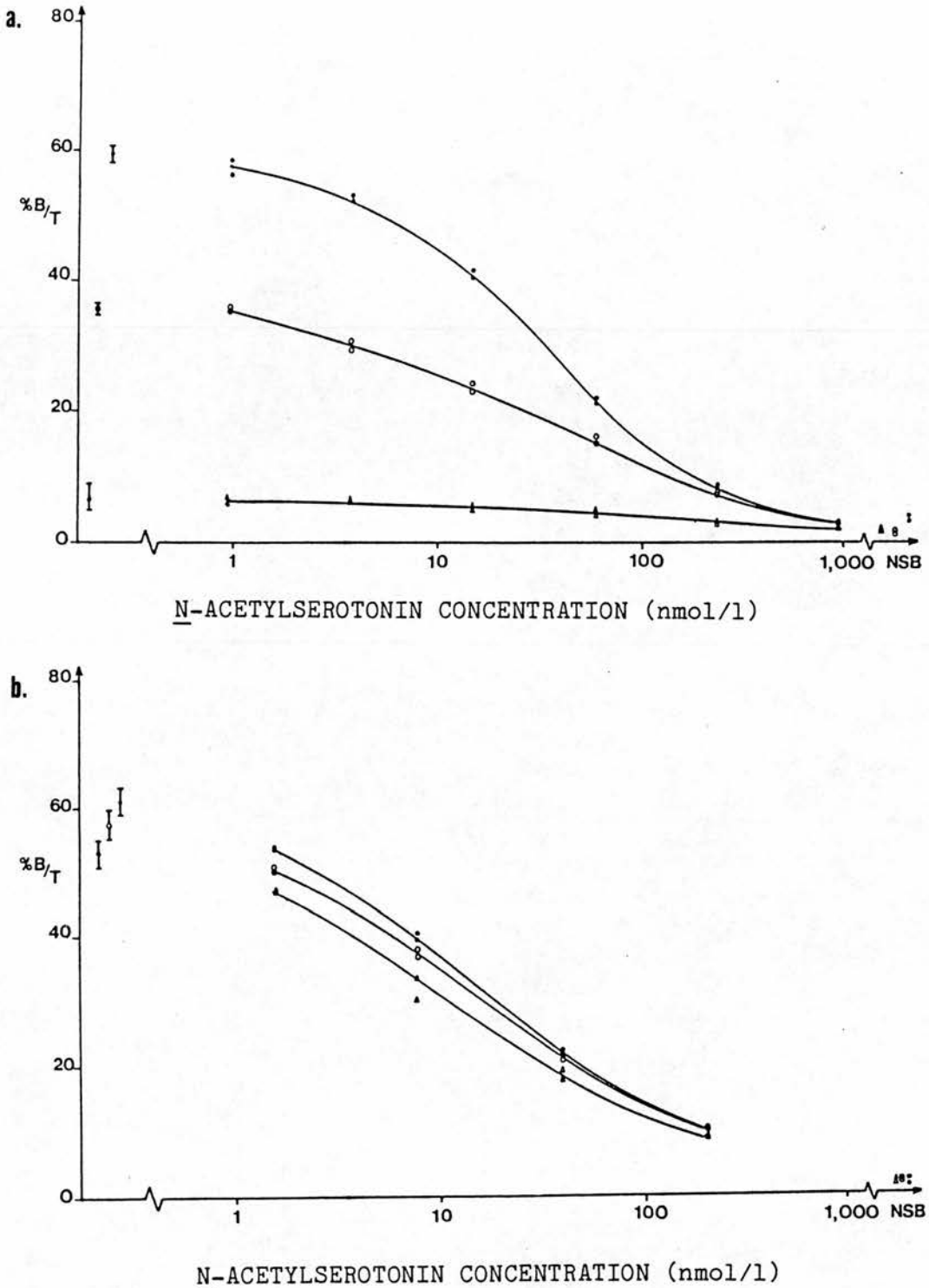


Figure 3.19 Effect of pH on the standard curve using a) guinea pig antiserum, or b) rabbit antiserum. B_0 = mean ± SD of triplicate estimations.

Δ——Δ = pH 4
0——0 = pH 5
●——● = pH 6.2

The tubes and contents were incubated at 4°C overnight either (a) after the addition of the NIRS and DARS, or (b and c) immediately after the addition of the mixture of primary antiserum, NIRS, and DARS; separation was as described above (3.3.4c).

There were no differences between the standard curves obtained under any of these conditions (Figure 3.20), and thereafter the sera were added as a single aliquot of a freshly prepared pre-mixture.

3.3.6 Acylation of Serotonin

3.3.6.1 Succinylation

In order to determine the appropriate amount of potassium hydroxide (KOH) to add to the succinylation mixture so that the final pH would be in the range 5-7, which would avoid oxidation of serotonin, but still enable hydrolysis of the 5-O-succinyl moiety (Means and Feeny, 1971), a fresh solution (500 µl) of serotonin (880 nmol/l) in 0.05 mol/l pH 7.4 buffer was added to succinic anhydride (20 and 30 mg) in glass tubes, and vortexed until the anhydride dissolved. Increasing volumes (0-90 µl) of KOH (4 mol/l) were added and the pH measured after vortexing. Initial results (Figure 3.21) indicated that with 20 mg of succinic anhydride, approximately 50 µl of 4 mol/l KOH were required to take the reaction mixture to pH 6, and so in a second experiment, a fresh solution of serotonin (440 pmol) in 0.05 mol/l pH 7.4 buffer (500 µl) was added to succinic anhydride (20 mg) in a glass tube, vortexed, and followed by the addition of 50 µl of KOH (4 mol/l). The tube was incubated overnight at 4°C, and analysed the following day. The yield of

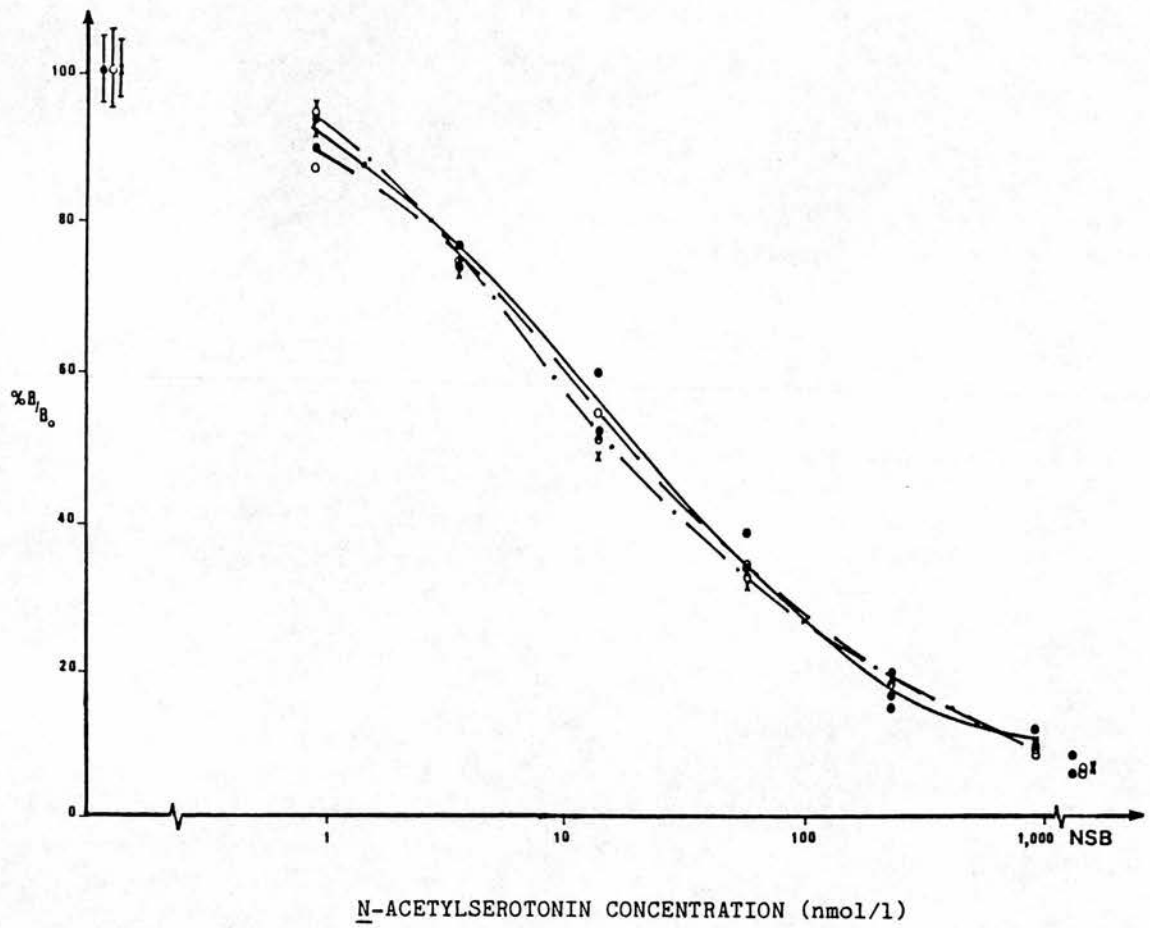


Figure 3.20 Effect of adding antisera separately (●——●), or as a pre-mix (O- - - -O), or as a pre-precipitate (X—·—X). B_0 = mean ± SD of triplicates.

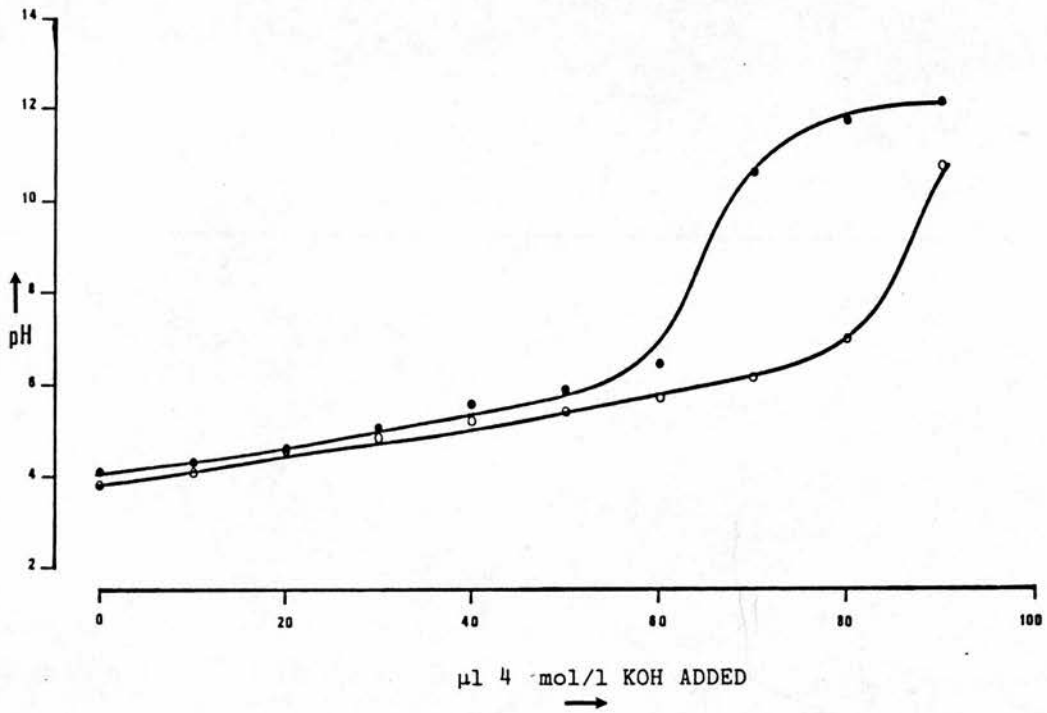


Figure 3.21 Effect of adding potassium hydroxide on the final pH of succinylated serotonin:

● = succinylation with 20 mg anhydride
O = succinylation with 30 mg anhydride.

N-succinamylserotonin as measured by HPLC, was 68.2%. There was no remaining unmodified serotonin.

3.3.6.2 Acetylation by Acetic Anhydride

Serotonin (2.88 nmol) in protein-free assay buffer (500 µl) was acetylated by adding triethylamine (10 µl) followed by acetic anhydride (5 µl), and incubation at room temperature for 40 and 90 min. Analysis by HPLC with UV detection at 254 nm showed that under these conditions, nearly all (91.1%) of the serotonin was converted into O,N,-diacetylserotonin, with no reversion to the N-acetyl form, even after 90 min at room temperature (Table 3.5).

3.3.6.3 Acetylation by N-Acetoxysuccinimide, (NAS)

NAS was prepared by the method of Treadway and Schultz (1976). For acetylation, NAS was dissolved in methanol (127.6 µmol/ml), and an aliquot (20 µl) dried down under nitrogen in a glass tube while warming gently (45°C). To this was added serotonin (2.88 nmol) in protein-free assay buffer (500 µl), the tube vortexed, and incubated at room temperature. Analysis by HPLC-ECD of samples withdrawn at varying times showed that a) the acetylation was complete after 40 min, and b) the N-acetylserotonin produced was stable for at least 3 h at room temperature (Figure 3.22). There was virtually no O,N,-diacetyl-serotonin produced using NAS (<5.1%; Table 3.5). Acetylation of 10 samples of serotonin by NAS as above resulted in a recovery of 80.0 ± 2.7% (mean ± SD) of the original mass of serotonin as N-acetylserotonin.

Sample	Mass O,N-diacetyl-serotonin (pmol/100 µl)	% equivalent of original serotonin
Serotonin	0	0
Serotonin/NAS (+40 min)	<15	<5.1
Serotonin/AA (+40 min)	549	91.1
Serotonin/AA (+90 min)	565	93.8

Table 3.5 Production of O,N-diacetylserotonin with acetic anhydride (AA), or N-acetoxysuccinimide (NAS).

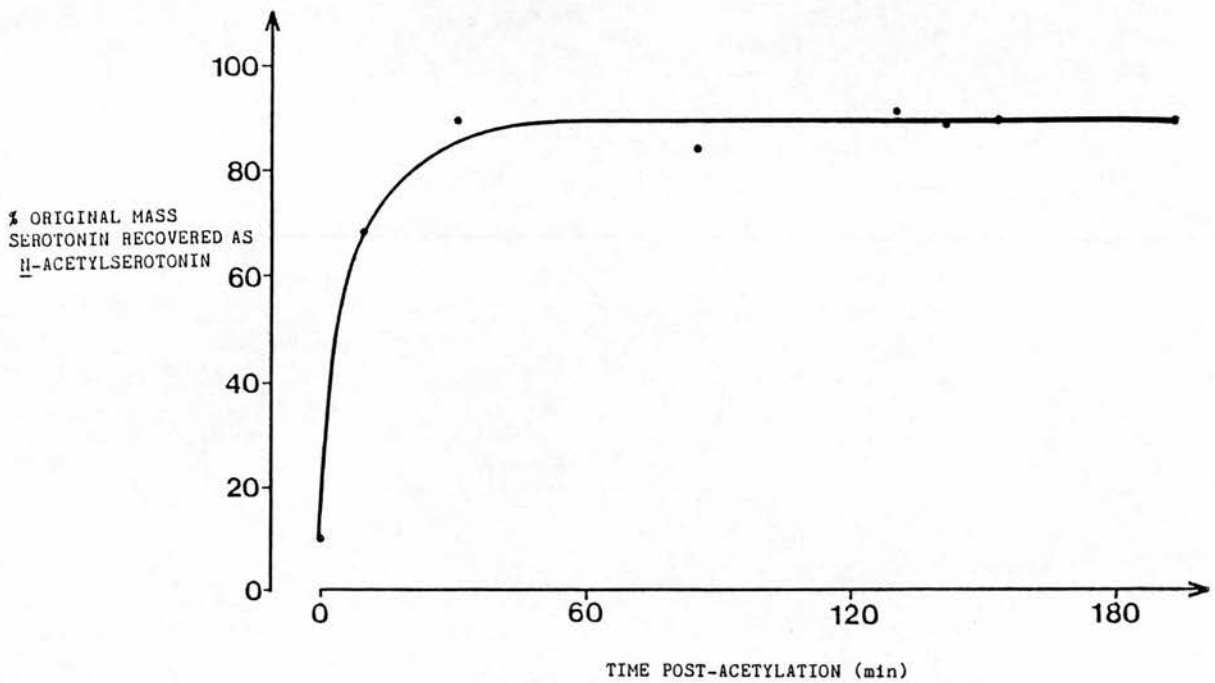


Figure 3.22 Time course of N-acetylation of serotonin by N-acetoxysuccinimide (NAS).

3.3.6.4. Neutralisation of Deproteinised Plasma

PRP was deproteinised as described for the HPLC-ECD assay (3.2.7) except no internal standard for recovery was used. Aliquots (600 µl) of the deproteinised PRP were dispensed into plain polystyrene tubes (10 ml), potassium hydroxide (0, 37.5, 75, 93.75, or 112.5 µmol) in water (150 µl) added, the tubes vortexed, and the pH measured.

The rise in pH which occurred after the addition of the potassium hydroxide is shown in Figure 3.23. The final pH was between 6 and 7.5 when 93.75 and 112.5 µmol respectively of KOH were added.

3.3.6.5 Acetylation by NAS after Deproteinisation and Neutralisation

PPP was spiked with serotonin (4 µmol/l final concentration), deproteinised as above (3.3.6.4), and aliquots (200 µl) of the supernatant added to NAS (1.59 µmol) dried down under nitrogen in glass tubes as above from a fresh methanolic stock (31.9 µmol/ml). The tubes were vortexed until the NAS was dissolved, an aliquot (50 µl) of the potassium hydroxide solutions (0, 12.5, 25, 31.25, or 37.5 µmol) added, the tubes vortexed, and left an hour at room temperature. The acetylated samples were assayed by HPLC-ECD for serotonin and N-acetylserotonin.

The maximum yield (85.2%) of N-acetylserotonin was obtained when 37.5 µmol of KOH were added to the acetylation mixture (Figure 3.24). With no added acetylating agent, 95.8% of the original mass of serotonin was recovered unmodified, and no peak corresponding to N-acetylserotonin was seen. When no KOH was added, the pH after 40 min incubation at room temperature was 4.8. At this pH, only 5.2% of

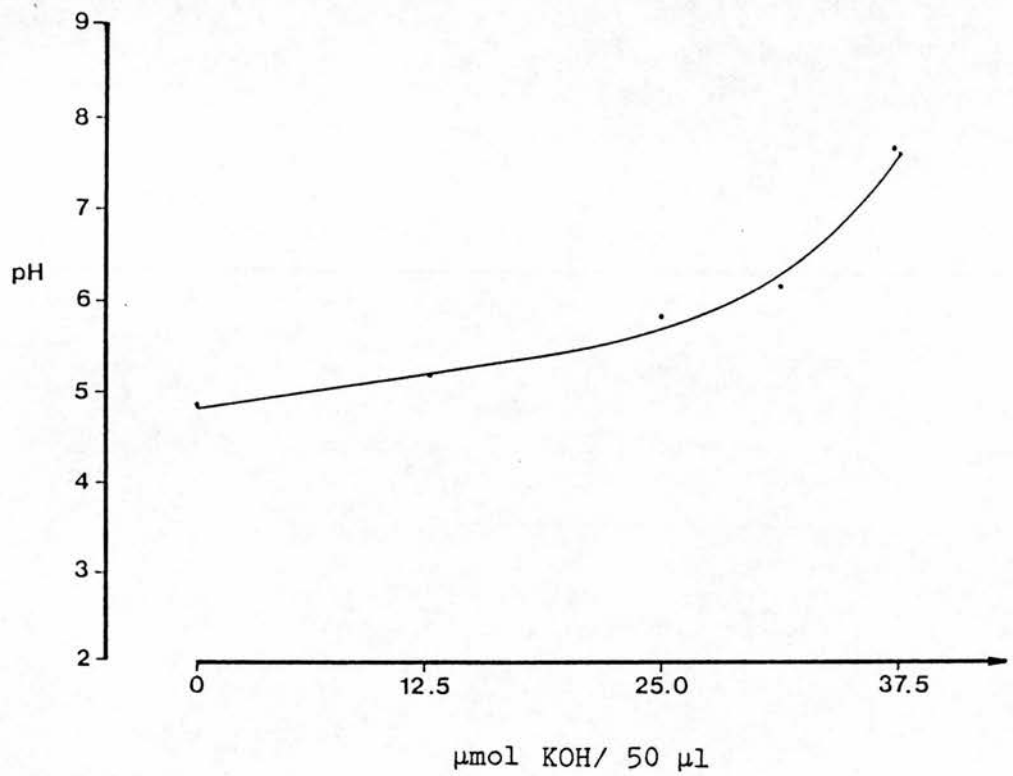


Figure 3.23 **Effect of adding potassium hydroxide on the pH of deproteinised PRP.**

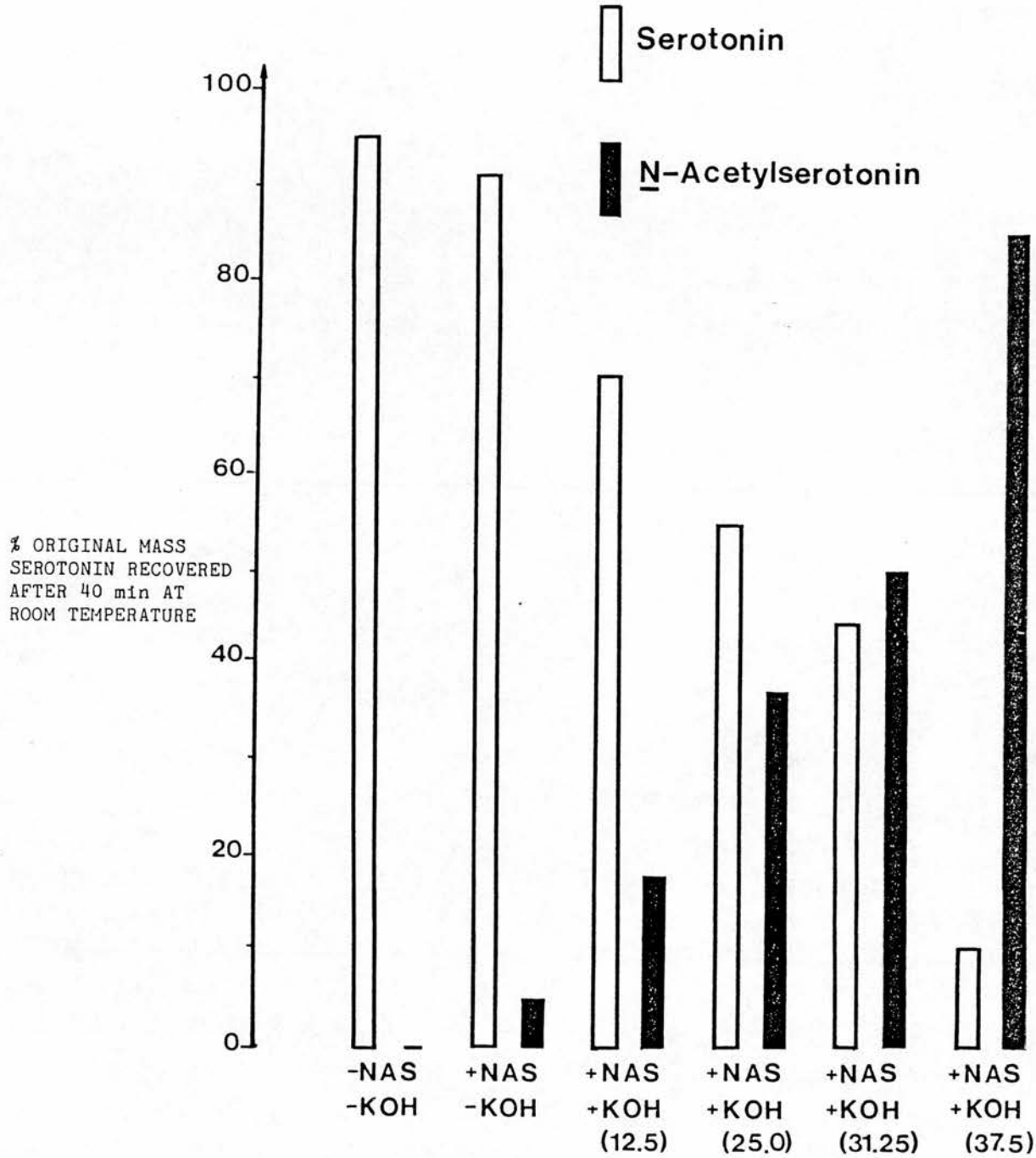


Figure 3.24 Acetylation of serotonin in PPP by NAS after deproteinisation and neutralisation with potassium hydroxide (value in brackets = µmol KOH added).

the original mass of serotonin used was recovered as N-acetyl-serotonin, and 91.7% was unmodified serotonin. When 37.5 µmol KOH were added in 0.5 mol/l protein-free assay buffer (pH 6.2), the final pH was 6.44 ± 0.08 (n=3). This mass of KOH (or the molar equivalent of NaOH) was subsequently used (diluted in 0.5 mol/l protein-free assay buffer, initial pH 6.2) to neutralise acidified, deproteinised plasma for acetylation.

3.3.7 Preparation of Charcoal-stripped PPP (CSPPP)

PPP was prepared from normal volunteers as above, and pooled. For charcoal stripping, the pooled PPP was spiked with purified [^{3}H]-serotonin and stirred for 4 h at 4°C with dextran coated charcoal (previously washed with 0.05 mol/l phosphate buffer, pH 7.4) in the ratio of plasma:charcoal:dextran of 100:10:1. The plasma was centrifuged at 1,720 x g for 30 min at 4°C to remove the bulk of the charcoal, and the supernatant filtered through a 0.22 µm filter to remove charcoal fines. An aliquot (100 µl) was taken for scintillation counting, and the remaining charcoal-stripped PPP (CSPPP) stored as aliquots of 20 ml at -20°C. The CSPPP was rendered 95.5 ± 3.3% free of added tracer after treatment as described (n=5).

3.3.8 RIA of Standard Curves with Acetylation of Serotonin by NAS after Deproteinisation and Neutralisation

Authentic serotonin was diluted either in assay buffer, or PPP/assay buffer (1/20), or CSPPP/assay buffer (1/20), to give a series of standards over the range 0-1,000 nmol/l. Each batch of PPP

was routinely checked for serotonin, and used only if the apparent concentration was <20 nmol/l, since this would be undetectable in the RIA after dilution. The standards (450 µl) were deproteinised as above (3.3.6.4), duplicate aliquots (2 x 200 µl) added to NAS (1.59 µmol) previously dried down in glass tubes, and vortexed. The solutions were neutralised with KOH (37.5 µmol) in 0.5 mol/l protein-free assay buffer (50 µl), vortexed, and incubated at room temperature for 60 min. Glycine (8.3 µmol) in assay buffer (50 µl) was added, the tube vortexed, and left a further 30 min. The standards were assayed by adding tracer (1 fmol, approximately 5,000 dpm) in assay buffer (100 µl), followed by a fresh mixture of primary antiserum, NIRS, and DARS in assay buffer (300 µl), at dilutions which would give final concentrations of 1/28,000, 1/4,800, and 1/240 respectively in the incubate (700 µl). Incubation was overnight at 4°C, followed by separation.

When plotted as percent tracer bound/percent tracer bound in the zero standard (i.e.% B/B_0), there was no apparent difference either between buffer and CSPPP curves, or between buffer and PPP standard curves (e.g. Figure 3.25a and b). A drop in the absolute value of the zero binding was observed with plasma, although this was not significant (Student's $\underline{t}$ test); if the mean zero binding in buffer was expressed as 100%, the corresponding values for CSPPP and PPP were 95.2 $\pm$ 11.6%, and 78.0 $\pm$ 20.2% respectively (n=4). Since there was no difference in the overall curve shape, but there was appreciable difference in the zero binding in the presence of plasma, in order to minimise this matrix effect, standard curves for assay of

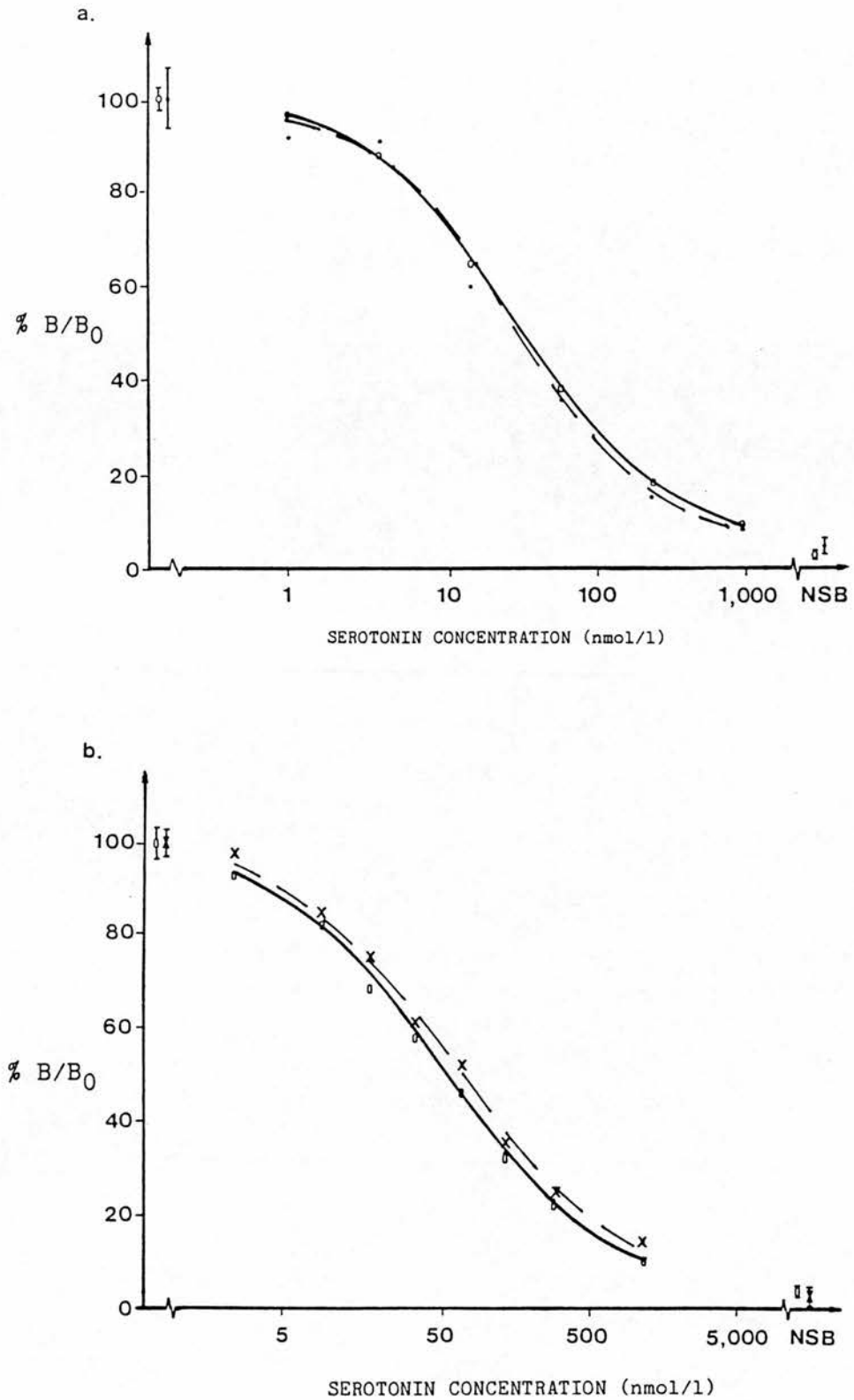


Figure 3.25 Comparison of buffer standard curve (O——O) with: a) 1/20 PPP in buffer (●- - -●), or b) 1/20 CSPPP in buffer (X- - -X). Points represent means of duplicate estimates; B_0 and NSB = means $\pm$ SD of triplicates.

human PRP were always constructed using serotonin prepared in normal PPP diluted 1/20 in assay buffer.

3.3.9 Analysis of Human PRP Samples by HPLC-ECD and RIA

For the HPLC-ECD assay, samples of PRP (400 µl) were deproteinised as above (3.2.7), then assayed by HPLC, previously calibrated with fresh dilutions of serotonin and internal standard. PRP samples for analysis by RIA were diluted in assay buffer (1/20), deproteinised, neutralised, acetylated and assayed as described above (3.3.8). The standards for the standard curve consisted of serotonin freshly diluted in a solution of PPP in assay buffer (1/20), to give a range of concentrations of 0-1,000 nmol/l. The standards were deproteinised, acetylated and assayed in parallel with the unknown samples during each RIA. The results obtained by HPLC-ECD and RIA were compared by linear regression analysis.

A representative standard curve and precision profile (Raab and McKenzie, 1981) derived from the mean data for eight consecutive PRP assays is shown in Figure 3.26. With incubation conditions as described above, mean zero binding from eight standard curves was 48.8 $\pm$ 6.1% of the total added radioactivity. The formal sensitivity of the standard curve (2.5 SD from the zero standard) was 2.0 nmol/l, or 0.40 pmol/tube (70.4 pg/tube), which after correcting for dilution, corresponds to a serotonin concentration in PRP of 40 nmol/l. Figure 3.27 shows the regression equation for comparison of serotonin values measured by HPLC-ECD and RIA. Of the normal subjects studied, PRP serotonin concentrations (mean $\pm$ SD) were

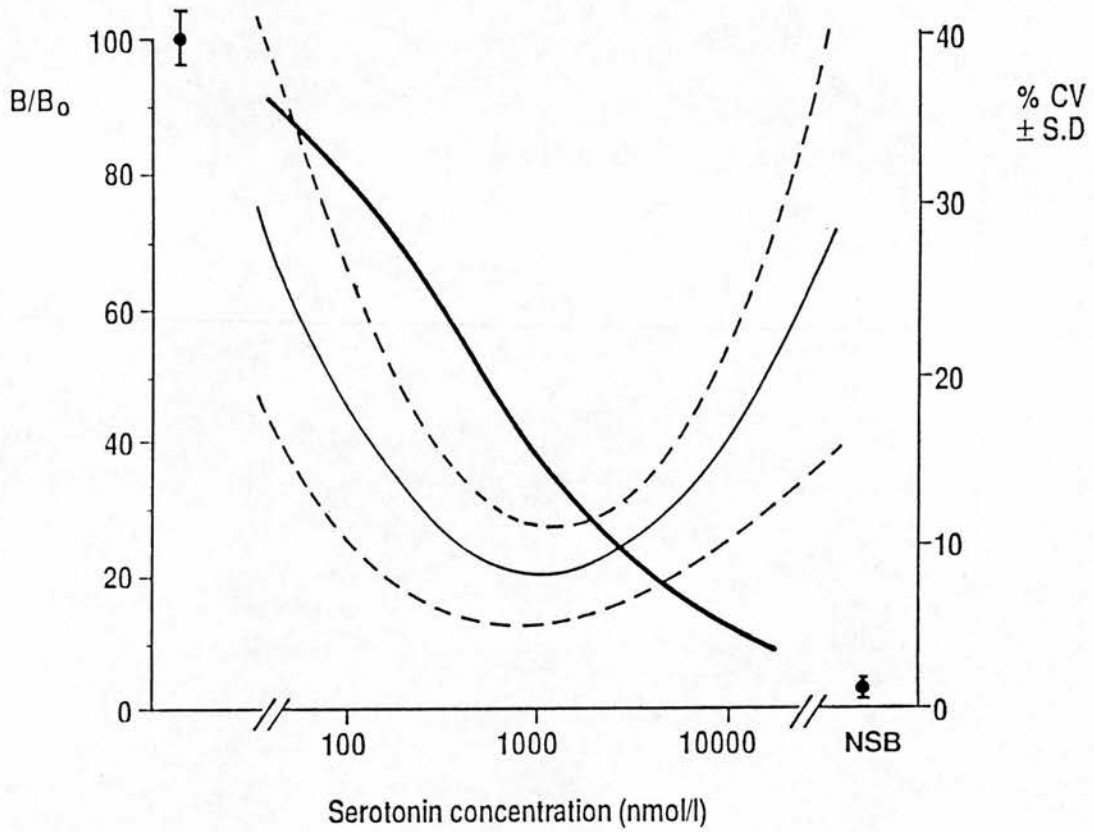


Figure 3.26 Standard serotonin curve for human PRP RIA (mean value ———), with the associated precision profile (mean value —— and SD - - -) for eight consecutive assays. Mean B_0 was 48.1 ± 6.1% of total added radioactivity. B_0 and NSB = mean values ± SD.

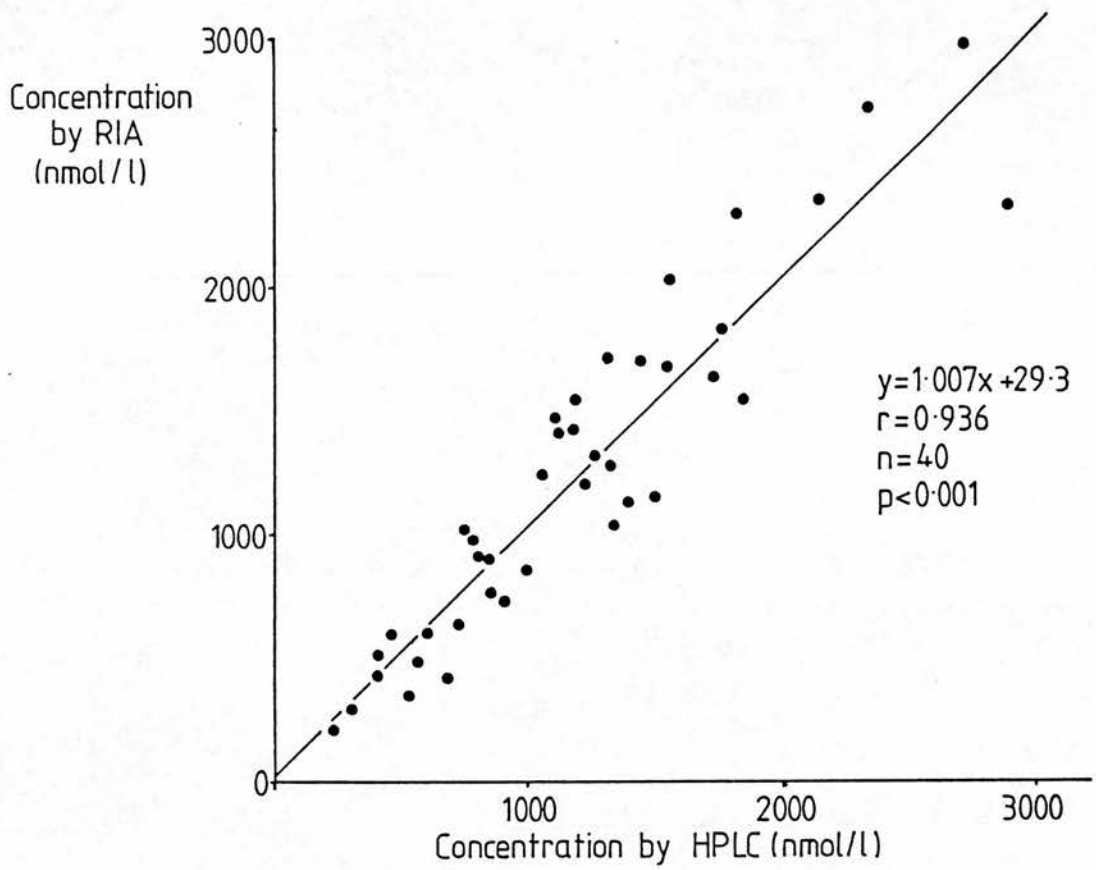


Figure 3.27 Correlation of PRP serotonin concentration as determined by HPLC and RIA. Standard error of slope = ± 0.061, standard error of intercept = ± 82.9. Fitted regression line not significantly different from y = x.

1548 ± 541 nmol/l for females (n=5), and 1404 ± 827 for males (n=6). This difference was not significant (Student's *t* test). No difference was seen between a standard curve which was assayed immediately after the acetylation had been performed, and the same standards when assayed after freezing for two days at -20°C (Figure 3.28).

Scatchard analysis of the standard curve (Scatchard, 1949) demonstrated that there was a population of antibodies in antiserum R16B4 with an affinity of 1.62×10^8 l/mol (Figure 3.29). This was taken to be the affinity constant of the antiserum.

3.3.10 Cross-reactivities

These studies were performed with the test indoleamines and standard serotonin diluted in assay buffer prior to deproteinisation. Cross-reactions were measured for the test compounds themselves, and, where appropriate, after *N*-acetylation as above (3.3.8). Percentage cross-reactivity was calculated from the ratio of the mass *N*-acetylserotonin at 50% B/B_0: mass of cross-reactant at 50% B/B_0.

The major (0.12%) formal cross-reactant (see Table 3.6) was unacetylated serotonin. *N*-Acetylation increased the cross-reactivity of the other indoleamines tested, but methylation of the 5-hydroxyl group of *N*-acetylserotonin reduced cross-reactivity from 100% to <0.02%.

3.3.11 Precision Studies Using PPP Spiked with Standard Serotonin

Serotonin was added to PPP prepared from normal volunteers, at final concentrations of 250, 750, and 2,500 nmol/l, and aliquots

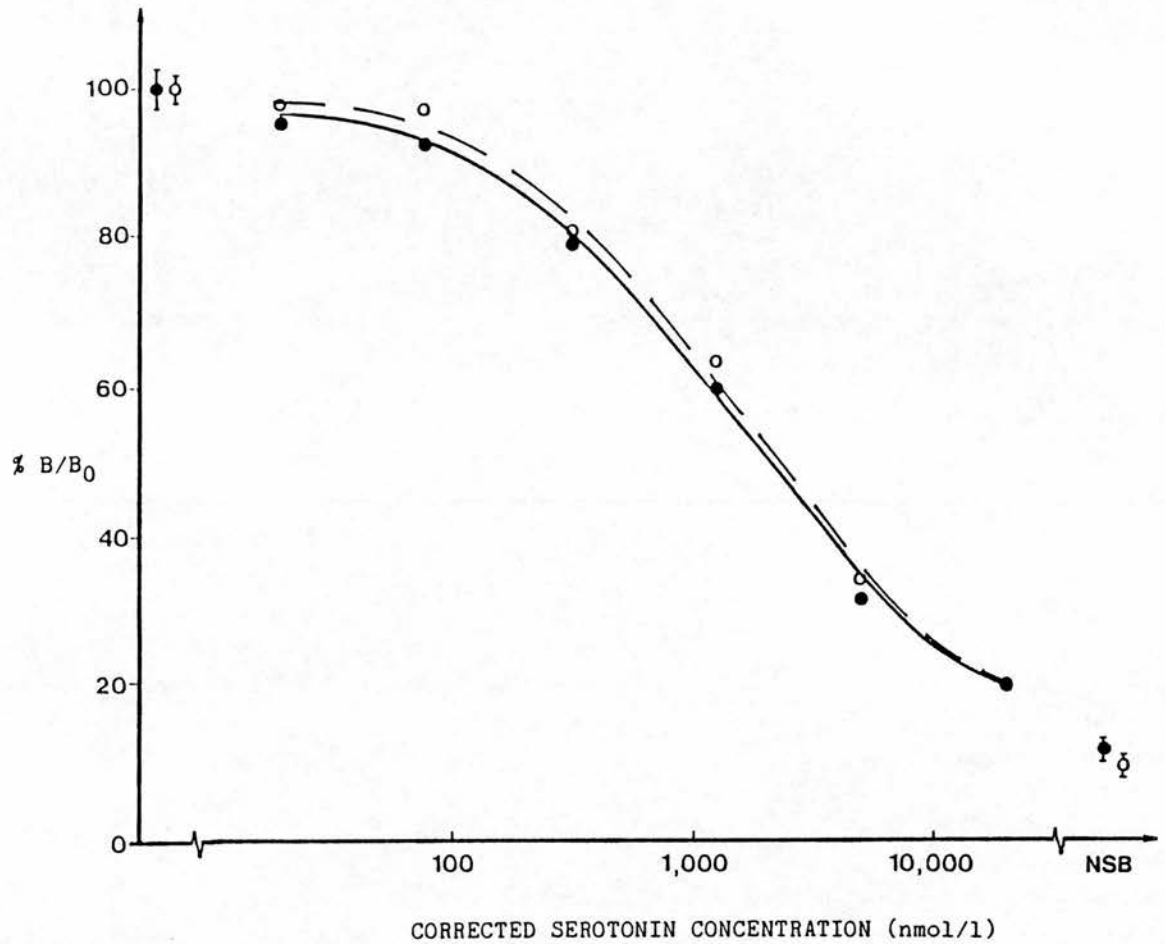


Figure 3.28 Plasma standard standard curves assayed either immediately (●-----●), or after storage at -20°C for 48 h (O- - -O). Points represent means of duplicate estimations; B_0 and NSB = means $\pm$ SD of triplicates.

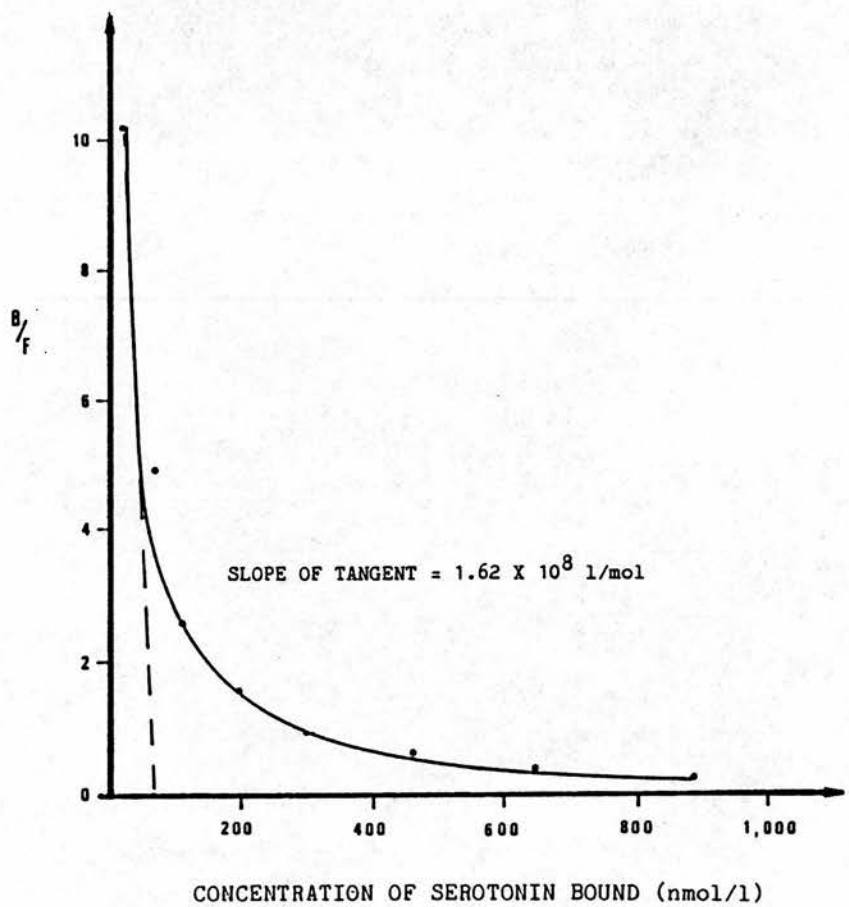


Figure 3.29 Scatchard analysis of the serotonin standard curve in buffer. Points = Mean values of 7 consecutive curves.

Compound	% Cross-reactivity
N-Acetylserotonin	100.0
5-Hydroxytryptamine (serotonin)	0.12
5-Hydroxyindoleacetic acid	<0.03
N-Acetyltryptamine	0.03
N-Acetyl-5-methoxytryptamine	<0.02
N-Acetyl-5-hydroxytryptophan	<0.01
5-Hydroxytryptophan	<0.01
5-Methoxytryptamine	<0.01
Tryptamine	<0.01

Table 3.6 Cross-reactivities of the R16 antiserum.

(845 µl) frozen at -20°C in polypropylene microcentrifuge tubes. These recovery pools were assayed by HPLC-ECD and RIA over a period of 8 weeks to determine intra- and inter-assay precision.

Intra- and inter-assay variation as calculated from repeat analyses of the spiked pools are shown in Table 3.7. Intra- and inter-assay variations in the HPLC-ECD assay were lower than in the RIA at all the tested concentrations, but the analytical recoveries of serotonin were similar for both assays. No decrease in the values obtained from the spiked pools was seen in either the RIA or HPLC-ECD assay over a period of two months.

3.3.12 Validation of RIA for Rat Serum

Comparative assay of serotonin standards prepared either in charcoal-stripped rat serum (prepared from neat serum diluted 1/10 in saline), normal human PPP, or charcoal-stripped rat "coagulant" serum, all diluted 1/20 in buffer, showed that the rat serum prepared by collection into saline alone shifted the lower concentrations of the standards the curve to the right (Figure 3.30). This indicated that the serotonin in the untreated rat serum curves may have been becoming degraded, and unable to displace the tracer bound to the antibodies. This effect was abolished by preparation of serum in "coagulant". Thus rat serum was prepared by diluting whole blood in a "coagulant" as described for the HPLC-ECD assay. The supernatant was assayed by HPLC-ECD and RIA and the results compared by linear regression analysis. The regression equation for samples measured both by HPLC-ECD and the RIA is shown in Figure 3.31.

	Recovery Pool (nmol/l)	HPLC $\bar{x} \pm$ SD	%CV	n	RIA $\bar{x} \pm$ SD	%CV	n
Intra-	250	252.0 ± 5.2	2.1	5	214.8 ± 22.5	10.5	10
assay	750	884.2 ± 5.7	0.6	5	773.6 ± 79.6	10.4	10
variation	2,500	2,579.0 ± 79.1	3.1	5	2,296.8 ± 131.1	5.7	10
Inter-	250	259.3 ± 14.8	5.7	12	242.4 ± 36.6	21.2	12
assay	750	836.9 ± 46.9	5.6	12	885.0 ± 106.6	12.0	12
variation	2,500	2,432.0 ± 158.8	8.2	12	2,465.5 ± 364.8	14.8	12
Inter-	250	103.8 ± 5.9	---	---	97.0 ± 20.6	---	---
assay	750	111.6 ± 6.2	---	---	118.0 ± 14.2	---	---
% recovery	2,500	97.3 ± 8.0	---	---	98.6 ± 14.6	---	---

Table 3.7 **Intra- and inter-assay variation for the RIA and HPLC-ECD assay.**

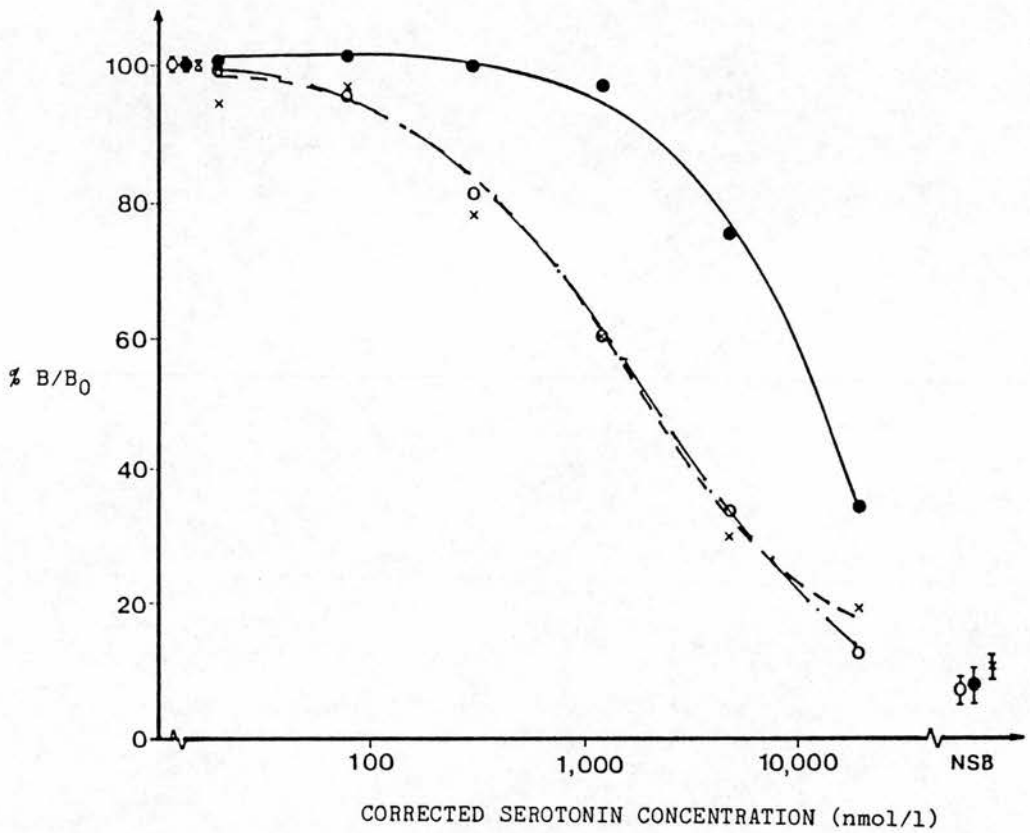


Figure 3.30 Effect of normal rat serum (●——●) or rat serum collected into the coagulant (X- - -X) on the RIA standard curve (normal human curve O—·—O). Points represent means of duplicate estimations; B_0 and NSB = means ± SD of triplicates.

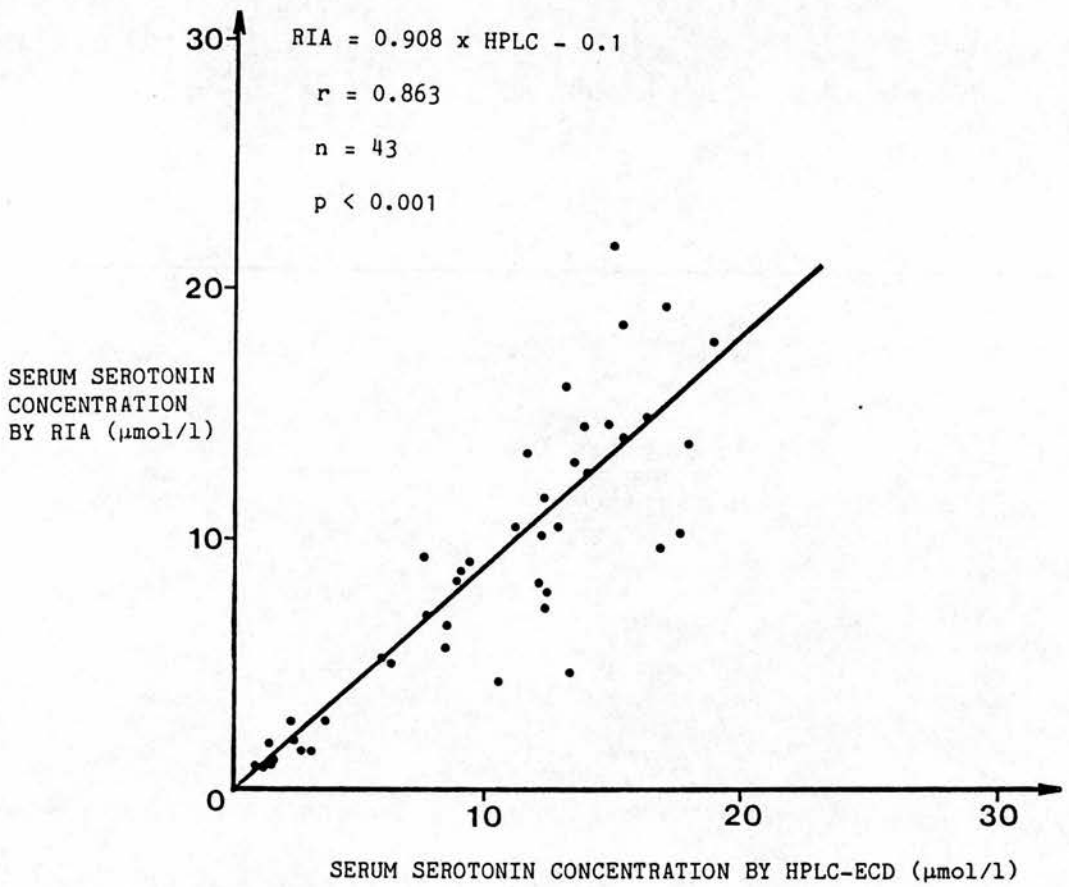


Figure 3.31 **Correlation between rat serum serotonin concentration as determined by RIA and HPLC-ECD assay.**

3.3.13 Validation of the RIA for *in vitro* Experiments

Samples of PRP (100 µl) were diluted in a mixture (900 µl) of Medium 199/ACD (1:9, pH 7.4), and 0.2 mol/l protein-free assay buffer (1 ml, containing 2 µmol/l chlorimipramine) was added to each of the diluted samples. Standards were made up in PPP diluted 1/10 in Medium 199/ACD (pH 7.4), followed by a 1/2 dilution with 0.2 mol/l protein-free assay buffer, which also contained 2 µmol/l chlorimipramine. Standards and diluted samples were assayed by RIA as described above, and the neat PRP samples were also assayed for serotonin by HPLC. The results obtained by the RIA and the HPLC-ECD as analysed by linear regression analysis are shown in Figure 3.32a, together with a typical *in vitro* standard curve (Figure 3.32b).

3.3.14 Analysis of PRP from a Patient with the Carcinoid Syndrome

Blood was collected from a patient diagnosed as suffering from the carcinoid syndrome, and PRP prepared as described (3.2.5). The PRP was assayed by RIA after a twenty-fold dilution in assay buffer, and a further two dilutions in PPP/assay buffer (1 part PPP/19 parts buffer).

The diluted PRP from the carcinoid patient was parallel to the standard curve (Figure 3.33), indicating that even plasma which contains grossly elevated levels of indoleamines has no detrimental effect on the assay.

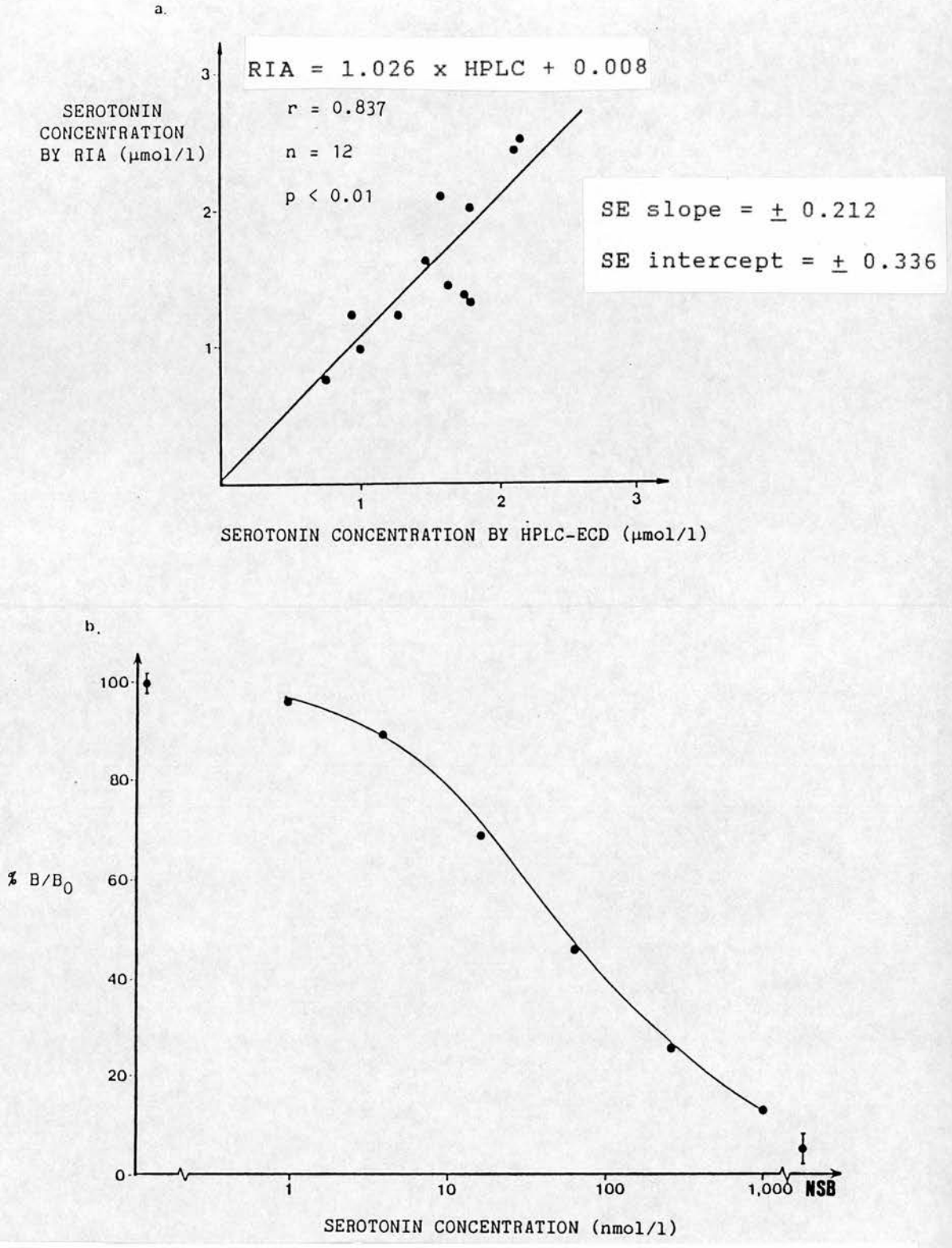
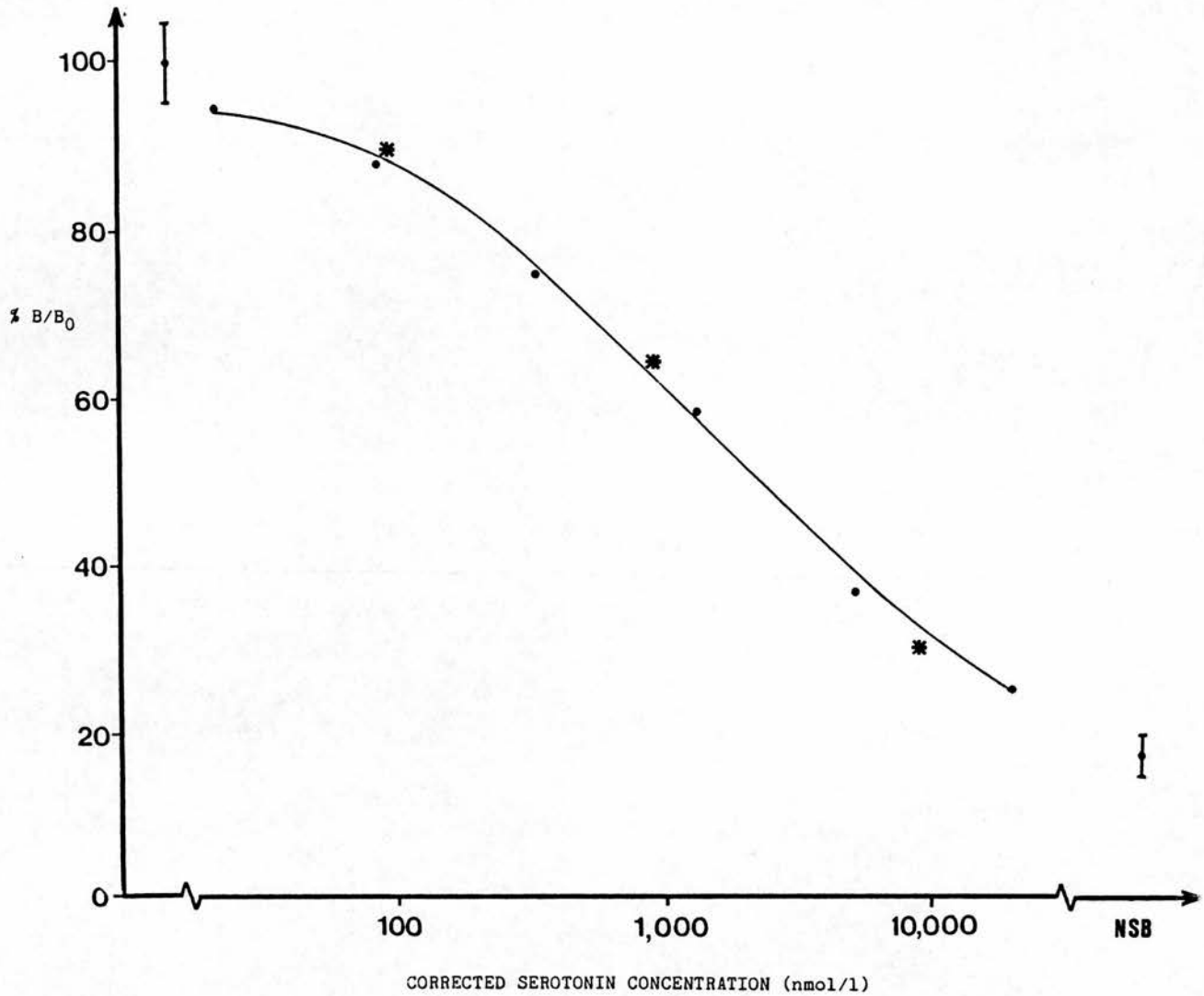


Figure 3.32 a) Correlation between <u>in vitro</u> RIA results and HPLC-ECD assay; fitted regression line not significantly different from y = x. b) A representative standard curve for use with <u>in vitro</u> experiments. Points represent means of duplicate estimations; B_0 + NSB represent means ± SD of triplicates.



* = Diluted carcinoid PRP

Figure 3.33 Dilution of carcinoid PRP compared with standard curve.

3.3.15 Effect of 5-Hydroxytryptophan or p-Chlorophenylalanine on the Serotonin content of Rat Serum.

Male Wistar rats (200-250 g) which had been cannulated via the carotid artery were injected intra-peritoneally on the third and fourth days after cannulation with either a) 1 ml normal saline, b) 1 ml saline containing 5-hydroxytryptophan (5HTP; 4 mg/ml), or, in a separate experiment, c) 1 ml saline containing p-chlorophenylalanine (PCPA; 80 mg/ml). On day 5, serum was prepared as described (section 3.2.6b), and analysed for serotonin by RIA.

Treatment with 5HTP significantly ($p<0.001$; Student's t test) raised the serum level of serotonin from (mean $\pm$ SD) 6.6 $\pm$ 3.5 µmol/l in the control group (n=15) to 13.6 $\pm$ 6.3 µmol/l in the experimental group (n=16; Figure 3.34a); conversely, the serum concentration of serotonin in the rats treated with PCPA fell significantly ($p<0.05$; Student's t test) from 9.2 $\pm$ 4.3 µmol/l in the control (n=17) to 6.6 $\pm$ 5.4 µmol/l in the experimental animals (n=17; Figure 3.34b).

3.3.16 Release of Serotonin from Human Platelets by Thrombin Stimulation.

PRP was prepared from a normal volunteer, diluted 1/5 in Medium 199/ACD, and aliquots (400 µl) incubated in triplicate in conical polypropylene microcentrifuge tubes for 20 min at 37°C in a shaking water bath (200 strokes/min), in the presence of thrombin dissolved in saline (200 µl) at final concentrations of 0, 0.05, 0.5, 5.0, and 50.0 units/tube. A set of "Total" tubes was also included, containing PRP (400 µl) and saline (200 µl). The tubes were removed into an ice/water slurry, and diluted 1 + 1 with cold (4°C) 0.2 mol/l

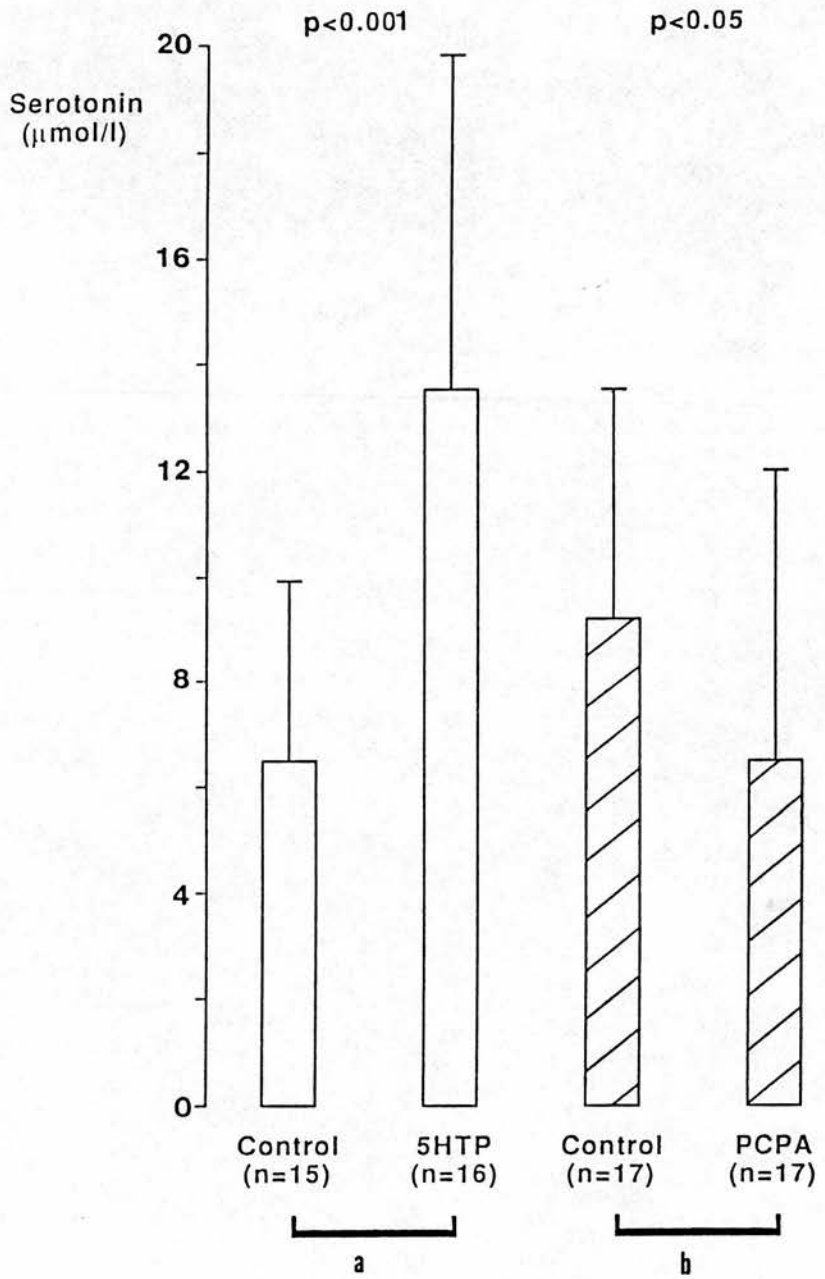


Figure 3.34 Effect of a) 5HTP and b) PCPA on rat serum serotonin (p value from Student's *t* test).

assay buffer, which also contained 2 µmol chlorimipramine/l. Then, except for the "Totals", which remained in the slurry throughout, the tubes were centrifuged for 60 s at 5,000 x g and 4°C. Aliquots (450 µl) of the supernatant from each tube (including 450 µl from the totals) were deproteinised, and assayed by RIA.

Incubation with 0.5, 5.0, and 50.0 units thrombin/tube resulted in a significant (Student's *t* test) dose-dependent rise in the serotonin concentration recovered from the supernatant (Figure 3.35).

3.4 Discussion

Although HPLC-ECD is currently the method of choice for analysis of serotonin, its major disadvantage is that comparatively few samples (<20) can be processed in a working day compared with the large throughput (>100 samples/day) of an established RIA, which would be necessary for a screening programme, or *in vitro* studies, where large numbers of samples may be generated in one experiment. The in-house HPLC-ECD system described in this thesis was developed and validated for serotonin a) to be used as an assay in its own right, b) to be used as a reference method for validation of the RIA, and c) to aid in the development of the RIA methodology (e.g. by monitoring the progress of the acylation experiments). The chromatographic conditions are similar to those used by other groups (e.g. Kamal, *et al.*, 1984a; Tagari, *et al.*, 1984), i.e. a reversed-phase ODS column, with a buffered mobile phase containing a small percentage of organic modifier, and a ion-pairing agent.

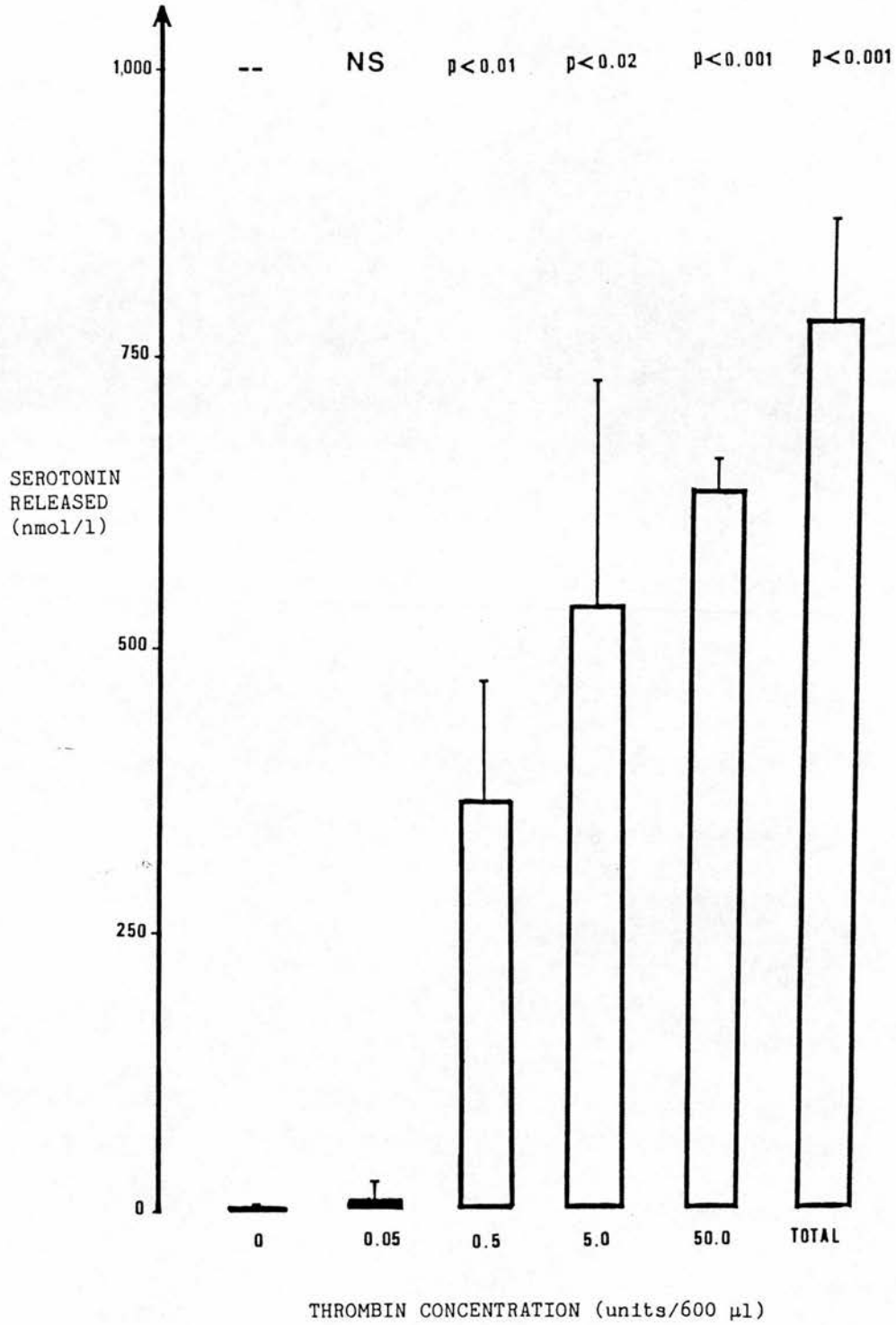


Figure 3.35 Effect of incubating PRP diluted in 199/ACD, with increasing doses of thrombin (mean values ± SD from triplicates, p values from Student's *t* test.

The experiments with the HPLC-ECD described demonstrate that:

a) the peak given by a pure standard solution of serotonin co-elutes with >90% of injected radioactivity from a fresh tritiated standard in which >90% of the activity is due to [^{3}H]-serotonin.

b) the response of the detector is linear over the tested range of standard serotonin concentrations (16-50,000 nmol/l), thus, since the regression line will pass through the origin (zero mass injected gives zero peak height), it can be expressed as the equation of a straight line:

$$y=mx + c$$

where y is peak height, x is mass of serotonin injected, m is the gradient of the line, and c is the intercept, which is zero. Therefore, a single injection of a known mass of pure standard can be used to calculate unknown masses by comparison of peak heights, since the gradient for standard and unknowns will be constant, therefore:

$$\frac{h_s}{m_s} = \frac{h_u}{m_u}$$

and

$$m_u = \frac{h_u \cdot m_s}{h_s}$$

where h_s and m_s are standard peak height and mass, and h_u and m_u are peak height and mass of the unknown respectively. The height of the internal standard is used in the same way to calculate the recovered mass, and therefore the recovery after deproteinisation.

c) Related indoleamines, although structurally similar to serotonin, exhibit different, characteristic retention times and oxidative voltammograms, when compared with serotonin. Unknown

peaks can therefore be identified as serotonin if they have the same retention time and oxidative voltammogram as a pure, standard solution.

Differential centrifugation is widely used in the isolation of platelets from whole blood (e.g. Sasa, et al., 1978; Tang and Frojmovic, 1980; Mezzano, et al., 1982; Kamal, et al., 1984a). Other methods have been used, whereby whole blood is spun through a density gradient of dextran (Launay, et al., 1982) or Percoll (Ding, et al., 1985a), and the platelets harvested. To separate the platelets from the gradient material, further centrifugation and washing steps would be required; the disadvantage of this is that the platelets are subjected to manipulation for longer than they are during a single centrifugation step, and this has been shown to result in release of platelet constituents (Parker, et al., 1984), or decreased platelet cAMP levels, possibly indicating an increase in platelet activation or aggregation (Gorman, et al., 1977), which could adversely affect any subsequent tests of function. Furthermore, release of platelet constituents during excessive centrifugation and washing would result in falsely depressed levels of serotonin, which is secreted during the platelet release reaction (Grette, 1962).

No attempts were made to use PPP in the assays to measure "free", or non-platelet bound serotonin, for several reasons. Firstly, PPP by definition is not devoid of platelet contamination; assuming a platelet serotonin concentration of approximately 300 pmol/10^8 platelets, and assuming 400 x 10^6 platelets/ml whole blood, a contamination of 1.0% after centrifugation would leave 4 x 10^6

platelets/ml PPP, which would correspond to an apparent serotonin level of 12 nmol/l. Only ultra-centrifugation is thought to be effective in removing residual, small platelets (Wolf, 1967). Contamination of PPP with platelets may arise from inefficient centrifugation, resuspension of platelets from the plasma/red cell interface, or from platelets becoming trapped in the layer of lipid floating on the top of the plasma (Rasi, 1979). Secondly, it is evident from studies of other products of platelet release (e.g. β-thromboglobulin, BTG), that the protocols used for blood sampling, centrifugation and storage are critical to avoid release of these platelet constituents (Ludlam and Cash, 1976; Levine, et al., 1981), whereas most published methods for production of PPP do not seem to take extensive precautions to avoid platelet activation during sampling. It has been suggested that the platelet α-granules which store BTG liberate their constituents more readily than the dense granules which store serotonin (Witte, et al., 1978), at least when stimulated by thrombin, hence it would seem that the precautions required when sampling for BTG are unnecessary for preparation of PPP for "free" serotonin analysis. Thirdly, the expected levels of "free" serotonin in carefully prepared PPP would seem to be <10 nmol/l, which approaches the cut-off limits for the working range both for HPLC-ECD and the RIA, making accurate and precise determinations difficult without prior sample concentration. It is presumably because of such difficulties that a large variation in "free" serotonin levels have been reported (Table 3.8).

No analysis of serotonin in whole blood was attempted, since

"Free" Serotonin (nmol/l)	Reference
87	Kellum and Jaffe, 1976
<7	Demet, et al., 1978
40	Frattini, et al., 1979
19	Koch and Kissinger, 1980
9	Engbaek and Voldby, 1982
72	Petrucelli, et al., 1982
17	Emson, et al., 1984
3.3-20.5	Hindberg, 1984
<10	Kamal, et al., 1984a
44	Tagari, et al., 1984
15	Artigas, et al., 1985
<1	Molyneux and Clarke, 1985

Table 3.8 Reported levels of serotonin in normal human PPP.

oxyhaemoglobin is liberated from the red cells during deproteinisation with perchloric acid, resulting in the rapid oxidation of serotonin, unless the blood is saturated with carbon monoxide prior to deproteinisation (Korpi, 1984). Also, when corrected for platelet count, no difference was seen in the serotonin content (on a per platelet basis) between whole blood and PRP (Kellum and Jaffe, 1976).

Serotonin is unstable at alkaline pH, and rapidly undergoes auto-oxidation in the presence of atmospheric oxygen (Garattini and Valzelli, 1965; Falkowski and Wei, 1981). Consequently, the monoamine is usually stored in acid, in the presence of an anti-oxidant, at 4°C (e.g. Sasa, et al., 1978; Fujimori, et al., 1982; Patthy and Gyenge, 1984; Tagari, et al., 1984). Under the conditions described above (3.2.1), standard serotonin is stable over a period of several months, and serotonin in deproteinised PRP samples stored at 4°C is stable throughout the course of an analytical run (3-4 h), which agrees well with published results (Guicheney, et al., 1985). Serotonin in plasma is also acceptably stable when stored as sealed aliquots frozen at -20°C, as demonstrated by the spiked PPP samples which were analysed over a period of two months. These spiked samples were not truly representative of PRP samples, as they did not contain platelets in appreciable concentrations, and platelets do contain a monoamine oxidase (MAO); however, the platelet MAO, MAO B, does not readily metabolise serotonin (Glover, 1977).

In the undiluted rat serum, there was a large variation in individual recoveries as measured by height of the internal standard

peak, and it was thought that this may have been due to either MAO activity, or a non-specific oxidation of serotonin by free oxyhaemoglobin in the serum (Korpi, 1984). When prepared using the "coagulant" containing a serotonin releasing agent (thrombin), a serotonin re-uptake inhibitor (chlorimipramine), and an inhibitor of monoamine-oxidase (pargyline), rat serum serotonin was stable for up to 2 h at 37°C, (Table 3.3) suggesting the degradation was due enzymatic activity rather than non-specific oxidation, since no anti-oxidant was added.

Acid deproteinisation is commonly used to remove proteins from plasma prior to analysis for serotonin (Rao, et al., 1976; Koch and Kissinger, 1980; Mefford, 1981; Tagari, et al., 1984). This is necessary both for the HPLC-ECD assay, and for the RIA; in the former, protein contamination of a column can rapidly lead to reduced resolution, increased back pressure, and generally impaired performance, and in the latter, free amino groups on the protein would compete with serotonin for the acetylating agent. Perchloric acid has been used in serotonin assays at final concentrations ranging from 0.05 mol/l (Meyer and Shoup, 1983) to 1 mol/l (Delaage and Puizillout, 1981). The concentration of perchloric acid used in the RIA (0.15 mol/l) should remove >75% of the protein (Blanchard, 1981). Acid deproteinisation has the added advantage that under these conditions (i.e. pH<6), serotonin is stable at room temperature (Garattini and Valzelli, 1965).

The significant difference between the recoveries of tritiated serotonin and the HPLC-ECD internal standard (Nω-methylserotonin)

from PRP may be due to impurities in the tracer, as it is not known exactly how the residual (<10%) of radioactivity behaves, i.e. whether it is distributed throughout the sample, or is concentrated in the supernatant, which would result in a falsely elevated recovery. Although it is assumed that the internal standard behaves in exactly the same way as serotonin during the deproteinisation, the fact that serotonin and the internal standard can be separated by HPLC-ECD underlines the point that they are not identical, and may possess different chemical properties.

Due to the intrinsic instability of serotonin at room temperature in the presence of atmospheric oxygen, and at neutral or alkaline pH, it was found necessary to synthesise the N-succinamylserotonin under nitrogen, as exposure to the alkaline hydroxylamine used to cleave the 5-O-succinyl moiety back to a 5-hydroxy group (Means and Feeny, 1971) otherwise resulted in the destruction of the indole nucleus. Similarly, the preparation of the immunogen had to be conducted in an inert atmosphere. The structures of serotonin and the acylated derivatives used in the RIA are shown in figure 3.36.

In attempting to produce a gamma-emitting tracer using the Bolton-Hunter reagent, it was hoped a) to increase the sensitivity of the assay by having a tracer with a bridge structure more similar to the modified antigen than to the original immunogen, (c.f. Corrie, et al., 1981), and b) to simplify the preparation of the tracer, since serotonin itself is used instead of the N-succinamyl derivative, and is acylated by the [^{125}I]-Bolton-Hunter reagent directly. This type of iodination is technically straightforward, and there seems no

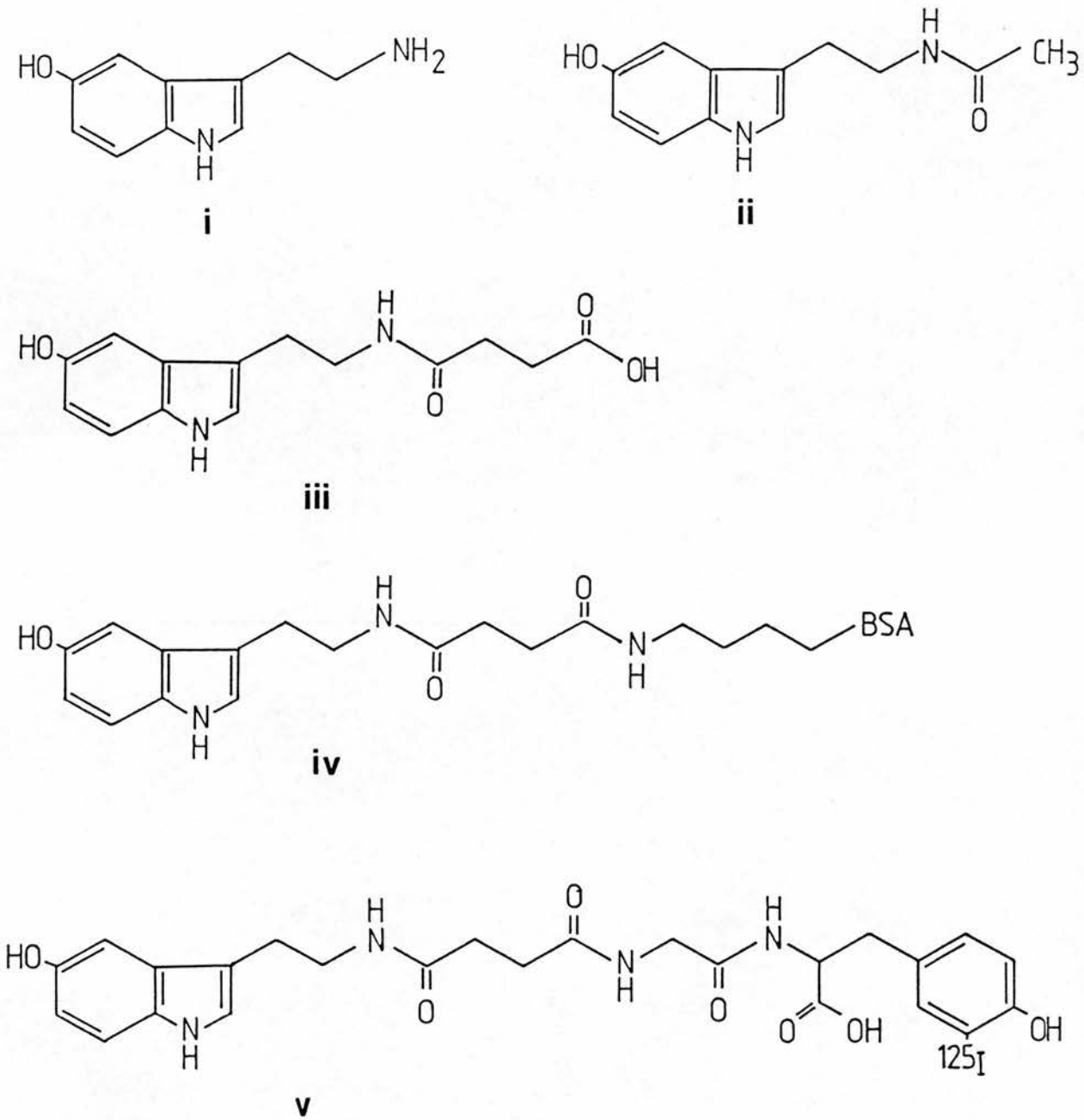


Figure 3.36 Serotonin and acylated derivatives:
i) serotonin

ii) N-acetylserotonin

iii) N-succinamylserotonin

iv) N-succinimylserotonin-BSA

v) N-succinamylserotonin-[^{125}I]-glycyl-tyrosine

obvious reason why virtually no binding of the tracer was seen, since a recent publication describes the preparation of such a tracer (Manz et al., 1985). It is possible that some auto-oxidation of serotonin occurred during the acylation step (pH 8.5), or during the thin-layer chromatography.

Separation of the bound fraction from the free was initially by ammonium sulphate precipitation as described by Kellum and Jaffe (1976), and after immunogenic activity was detected, dextran-coated charcoal (Al-Dujaili and Edwards, 1981) was used. These two separation systems were used to monitor activity in the bleeds, but were not used for the routine assay for several reasons: the precipitation of immunoglobulins by 50% saturated ammonium sulphate was extremely time-consuming, since the ammonium sulphate was added to each tube dropwise whilst vortexing; furthermore, the process had to be repeated after the first centrifugation to wash the pellet, otherwise the NSB was unacceptably high (>10%). Although separation using dextran-coated charcoal is widely used, it is far from ideal. Charcoal separation is sensitive to changes in pH, temperature and time (Ratcliffe, 1974; Hunter, 1978), and the possibility exists that the removal of the free tracer by the charcoal may alter the equilibrium of the binding reaction during separation (Thorell and Larson, 1978). Double-antibody separation has very little effect on the binding of tracer to the first antibody (Thorell and Larson, 1978), but does involve the risk of adding external analyte contained in the second antibody or carrier. With the serotonin RIA described above, this problem does not exist as such, since the assay measures

N-acetylated serotonin, and the acetylation reaction is completed prior to the addition of any of the antisera. Circulating N-acetylserotonin has been described in sera from rats, hamsters, and rabbits, but all reported levels have been less than 100 nmol/l (Pang, et al., 1980; Pang, et al., 1981; Geffard, et al., 1982; Pang et al., 1983), which would not be detectable in the assay system after dilution of the second antibody or carrier (typically 1/50 and 1/500 respective initial dilutions).

Immunisation of experimental animals with the N-succinamyl derivative of serotonin led to the production of antisera with such a high avidity for the bridge portion of the hapten, that the unmodified molecule is nearly 1,000 times less effective at displacing the tracer than the modified analyte. Similar effects have been described in earlier derivatisation assays for serotonin (Delaage and Puizillout, 1981; Geffard, et al., 1982), and also for the cyclic nucleotides (Cailla, et al., 1973; Fransden and Krishna, 1976).

Substitution of the side-chain of serotonin has slight effects on the stability of the molecule, as can be seen from the oxidative voltammograms (Figure 3.6); the further the curve lies to the right, the greater is the potential difference required to achieve oxidation (i.e. the greater the stability). Substitution of the 5-O group of serotonin markedly increases the stability of the molecule: 5-methoxy-N-succinamylserotonin can be exposed to the oxidising agent chloramine-T during an iodination, without the molecule undergoing oxidation (Geffard, et al., 1982), whereas 5-hydroxy-N-succinamyl-serotonin is rapidly degraded (Delaage and Puizillout, 1981). On

the basis of this observation, it seemed unlikely that 5-methoxy-*N*-acetyltryptamine (melatonin), or any of the 5-*O*-acyl derivatives would be detected by the electrochemical detector under the oxidative conditions used for serotonin and the other side-chain derivative indoleamines, hence the need to use UV detection for analysis of the diacetyl products of acetylation.

The lack of shift of the standard curve (when expressed as % B/B_0) with increasing tracer mass confirms that the tracer is of high specific activity (Hunter, 1983), therefore the decrease in zero binding is not due increasing tracer mass, but rather to some non-specific effect. Both previous publications which use this method of iodination (Delaage and Puizillout, 1981; Geffard, *et al.*, 1982) report the production of tracers with specific activities similar to that of the original sodium ^{125}iodide, i.e. >2,000 Ci/mmol.

The succinylation conditions used were similar to those employed in the original paper by Delaage and Puizillout (1981); the acetylation (and, in the production of a tritiated tracer, succinylation) conditions were essentially those used in cyclic nucleotide assays (Cailla, *et al.*, 1973; Fransden and Krishna, 1976). Succinylation by succinic anhydride, or acetylation by acetic anhydride were not pursued further, since both acylating agents require alkaline conditions for acylation (Means and Feeny, 1971), both result in the production of a 5-*O*-acyl group as well as the *N*-acyl derivative, and (as in the original immunogen), the 5-*O*-acyl group must be cleaved to avoid a loss in assay sensitivity (Cailla, *et al.*, 1973). Specific *N*-acetylation by *N*-acetoxysuccinimide

obviates this step, and therefore reduces the risk of auto-oxidation, since the pH is maintained below neutrality throughout. Others (Treadway and Shultz, 1976; Baumann, et al., 1973) have used N-acetoxysuccinimide as a specific N-acetylating agent, and the present work confirms this.

The sensitivity of the RIA for human PRP is comparable both with that of previously published RIAs (Table 3.9), which use either tritiated serotonin or gamma-emitting tracers, and also with that of HPLC-ECD, without extraction (approximately 2-5 nmol/l), although the precision with the RIA is poorer than the HPLC-ECD at the tested concentrations. This may be partly due to the use of an internal standard as a recovery marker in the HPLC-ECD assay, since this compensates for imprecision incorporated in sample preparation and analysis.

As expected, the precision in the the RIA is poorest at the upper and lower asymptotes, since at these flattening portions of the curve, small deviations in the measured response will be transposed into large variations in the apparent concentration (Ekins, 1974). The agreement between the RIA and the HPLC-ECD reference method shows that in human PRP, the endogenous concentration of N-acetylserotonin is negligible compared with serotonin (the HPLC-ECD separates these two compounds, allowing them to be measured independantly). This agrees with a previous observation (Hindberg, 1984), but other workers (Manz, et al., 1985) have found apparent N-acetylserotonin concentrations of 0-150 nmol/l in human serum, which is approximately 0-10% of the total serotonin concentration. The intercept from the

Immunogen	Tracer	Antiserum Dilution	Detection Limit (nmol/l)	Reference
Serotonin-*p*-amino-phenylalanine-BSA	[^{3}H]-5HT	1/50	10	Peskar & Spector, 1973
"	"	1/100	8	Kellum & Jaffe, 1976
"	"	1/300	2	Engbaek & Voldby, 1982
N-succinamyl5HT-HSA	[^{125}I]GT-5HT-HS	1/10,000	3	Delaage & Puizillout, 1981
N-succinamyl5MT-HSA	[^{125}I]GT-5MT-HS	1/200,000	5	Geffard, *et al.*, 1982
N-succinamyl5MT-BSA	[^{125}I]BH-5HT	1/3,000	12	Manz, *et al.*, 1985

BSA = bovine serum albumin

HSA = human serum albumin

5HT = 5-hydroxytryptamine, serotonin

5MT = 5-methoxytryptamine

5HT-HS = *N*-succinamylserotonin

5MT-HS = *N*-succinamyl-5-methoxytryptamine

[^{3}H]-5HT = tritiated serotonin

[^{125}I]GT = ^{125}Iodinated glycyl tyrosine

[^{125}I]BH = ^{125}Iodinated Bolton-Hunter reagent

Table 3.9 Sensitivities of previous serotonin radioimmunoassays

regression equation (29.3 nmol/l) is approximately 2% of the mean normal PRP serotonin levels, and may reflect true background N-acetylserotonin, or general "noise" due to assay imprecision. On the basis of the observed correlation, it has been assumed that compared with the levels of serotonin in human PRP, the circulating levels of N-acetylserotonin are negligible; this assumption is also tacitly implied in most descriptions of radioenzymatic assays (Walker, et al., 1983; Hammel, et al., 1978), and two other RIAs (Delaage and Puizillout, 1981; Geffard, et al., 1982). The rat serum RIA correlates well with HPLC-ECD analysis, although our levels of serotonin in normal rats are higher than other previous reports (Table 3.10). It is possible that our method of sample processing is less detrimental to endogenous serotonin than published methods, which use no enzyme inhibitors, and collect the blood neat (e.g. Ho, et al., 1975; Halvey, et al., 1980). The extra dilution step used in the collection of the serum will almost certainly introduce precision errors, and this may explain the large scatter seen on the regression plot. The increase in rat serum serotonin after injection with the serotonin precursor 5HTP, and the corresponding decrease following administration of the tryptophan hydroxylase inhibitor PCPA, demonstrate the expected physiological changes in serotonin levels can be detected by the RIA.

The correlation between the RIA using in vitro reagents suggest that the incubation medium (199/ACD) and chlorimipramine have no effect on the assay. The release experiment shows that serotonin released from platelets following thrombin stimulation can be

Serotonin (nmol/l)	Reference
242	Halvey, et al., 1980
443	Geffard, et al., 1982
4,000	Ho, et al., 1985
1,200	Marcenac and Blanche, 1985

Table 3.10 Reported serotonin levels in normal rat serum.

detected using the RIA, and that this release follows a dose-dependent pattern. Previous studies of the release of platelet serotonin *in vitro* have used a) platelets pre-loaded with either [^{3}H] or [^{14}C]-serotonin (e.g. Grant and Zucker, 1979), b) a complex, direct electrochemical detection method (Marcenac and Blache, 1985), or c) detection of released serotonin by HPLC analysis (e.g. Fujimori, *et al.*, 1982; Ingebretsen, *et al.*, 1985). All these methods have major disadvantages: the isotope method (a) requires pre-labelling of the platelets followed by liquid scintillation counting with its inherent disadvantages; furthermore, given that serotonin tracers may be nearer 50% than 100% pure for radioactive serotonin (see Section 3.2.7.1), the results may become difficult to interpret. Direct electrochemical detection or conventional HPLC (b and c respectively) both require expensive equipment, and the rapid throughput of large numbers of samples is not possible. The RIA, however, provides a relatively simple and cheap method for the rapid analysis of large numbers of *in vitro* samples.

The values obtained from normal volunteers are in good agreement with published serotonin levels (Table 3.11), and although the difference between the males and females was not significant as tested by the Student's *t* test, similar findings have been reported in the literature (Engbaek and Voldby, 1982; Le Quan-Bui, *et al.*, 1984; Manz *et al.*, 1985).

Serotonin (nmol/l)	Serotonin (pmol/10^8 platelets)	Reference
--	284	Born and Gillson, 1959
1,158	190	Kellum and Jaffe, 1976
690*	--	Demet, et al., 1978
809**	--	Sasa, et al., 1978
689	260	Frattini, et al., 1979
--	280	Ahtee, et al., 1981
1,136*	--	Anderson, et al., 1981
634*	--	Emson, et al., 1984
--	360	Hindberg, 1984
--	165	Kamal, et al., 1984a
500-1,700*	--	Korpi, 1984
1,160	--	Le Quan-Bui, et al., 1984
--	300	Artigas, et al., 1985
--	320	Ingebretson, et al., 1985
190-3,430	--	Manz, et al., 1985
--	259	Sharma, et al., 1985

* = whole blood
** = serum
Remainder are PRP

Table 3.11 Serotonin levels reported in normal humans.

Chapter 4

Platelet Serotonin Uptake and Aggregation Methods

4.1 Introduction

The preceding chapter has dealt with the development of an HPLC assay and an RIA for serotonin; this chapter describes the development of two tests of platelet function used in this thesis, viz. the uptake of [^{3}H]-serotonin by platelets, and platelet aggregation. By necessity, the former function test required detailed investigation, since, mainly due to the diversity of methods described in the literature and the apparent lack of standard conditions used, there is a wide variation in the magnitude of uptake parameters reported. In contrast, the technique of *in vitro* platelet aggregation is relatively simple, and the methodology has remained largely unchanged since the original description 25 years ago (Born, 1962).

4.2 Development of an *in vitro* Serotonin Uptake System

4.2.1 Collection of Blood

Platelet-rich plasma (PRP) was prepared from whole blood collected into acid/citrate/dextrose using a 19 gauge needle with minimal stasis as described earlier (section 3.2.5). Unless otherwise stated, blood was collected between 0900 h and 1000 h from a seated subject who had not received any drugs with known anti-platelet activity within the preceding seven days. Platelet numbers were estimated in a preliminary experiment either by diluting PRP 1/50 in a solution (1%) of ammonium oxalate in water, and counting in a haemocytometer, or by sending an aliquot of PRP (500 µl) in a standard (5 ml) potassium EDTA tube to the Haematology Department,

Western General Hospital, for analysis by Coulter counter.

Figure 4.1 shows a) the distribution of platelets in a sample of citrated whole blood, compared with b) the distribution of platelets in citrated PRP. The precision of counting platelets visually in a haemocytometer was poorer than by electronic particle (Coulter) counting (% coefficient of variation 15.2 and 1.2 respectively; Table 4.1); furthermore, the values obtained from the visual counts were higher than those obtained from the Coulter counter, but the difference was not significant ($p>0.05$, Student's t test). In all subsequent studies, platelet counts were determined by Coulter counter.

4.2.2 Optimisation of Centrifugation Conditions Required to Pellet Platelets

Fresh PRP (5 ml) was incubated with [^{3}H]-serotonin (approximately 30 pmol freshly diluted in saline (1.25 ml)), for 1 h at 37°C. Aliquots of this labelled PRP (300 µl) were dispensed into polypropylene microcentrifuge tubes, followed by 1 ml cold (4°C) chlorimipramine in saline (1 µmol/l). Triplicate sets of tubes were centrifuged for either 40, 90, or 180 seconds at 200, 500, 1,000, 2,000, 5,000, 10,000, or 20,000 x g, all at 4°C. The supernatant was aspirated from each tube, and the platelet pellet digested with a solution (500 µl) of ammonia in water (3% v/v) overnight at room temperature. An aliquot (200 µl) was taken from each tube for scintillation counting, and the mean recovered radioactivity for each triplicate point expressed relative to applied RCF (x g) for each centrifugation time used.

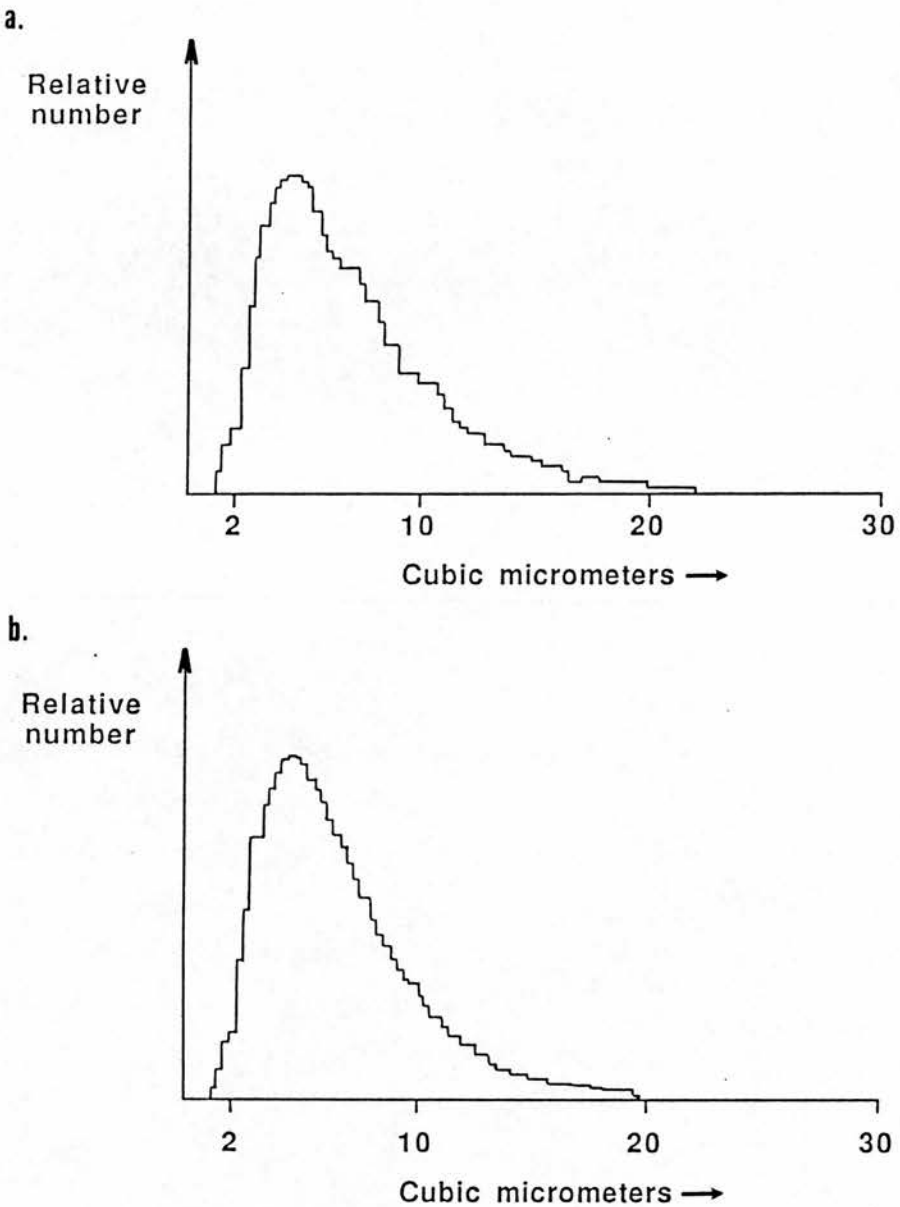


Figure 4.1 Comparison of profiles of platelet volume as determined by Coulter counter using platelets from either a) citrated whole blood or b) PRP prepared from citrated whole blood from a normal human volunteer (male).

Parameter	Visual Count	Coulter Count
Platelet Count (x 10^9/l)	736 $\pm$ 112	610 $\pm$ 7
CV (%)	15.2	1.2
p	NS	

Table 4.1 **Comparision of platelet count as determined from triplicate estimations of the same sample of PRP either by haemocytometer, or by Coulter Counter (mean value + SD, p value from Student's *t* test).**

The results (Figure 4.2) indicate that for all the tested centrifugation times, maximal recovery of radioactivity was achieved at RCF values of >2,000 x g. The centrifugation conditions used hereafter for serotonin uptake experiments were 1 min at 5,000 x g and 4°C.

4.2.3 Optimisation of Time Taken to Digest Platelet Pellet

Fresh human platelets were incubated with [^{3}H]-serotonin as described above, and six aliquots (6 x 300 µl) pipetted into separate polypropylene microcentrifuge tubes, followed by 1 ml of a cold (4°C) solution of chlorimipramine in saline (1 µmol/l). The tubes were centrifuged for 1 min at 5,000 x g and 4°C, and the supernatants aspirated. A solution (500 µl) of ammonia in water (3% v/v) was added to each tube, and after vortexing, each tube was left for either 0, 10, 20, 40, 60, or 80 min at room temperature before removal of a single aliquot of 200 µl for liquid scintillation counting.

As can be seen from Figure 4.3, maximal recovery of radioactivity was achieved after 40 min digestion, with no increase at up to 80 min at room temperature. Consequently, in all further uptake experiments, platelet pellets were digested for at least 40 min at room temperature prior to sampling for liquid scintillation counting.

4.2.4 Effect of Tracer Purity on Uptake

As demonstrated earlier (see 3.2.7.1), the [^{3}H]-serotonin is unstable once opened, and purity rapidly declines over a period of several weeks. In order to establish the effect of tracer purity on

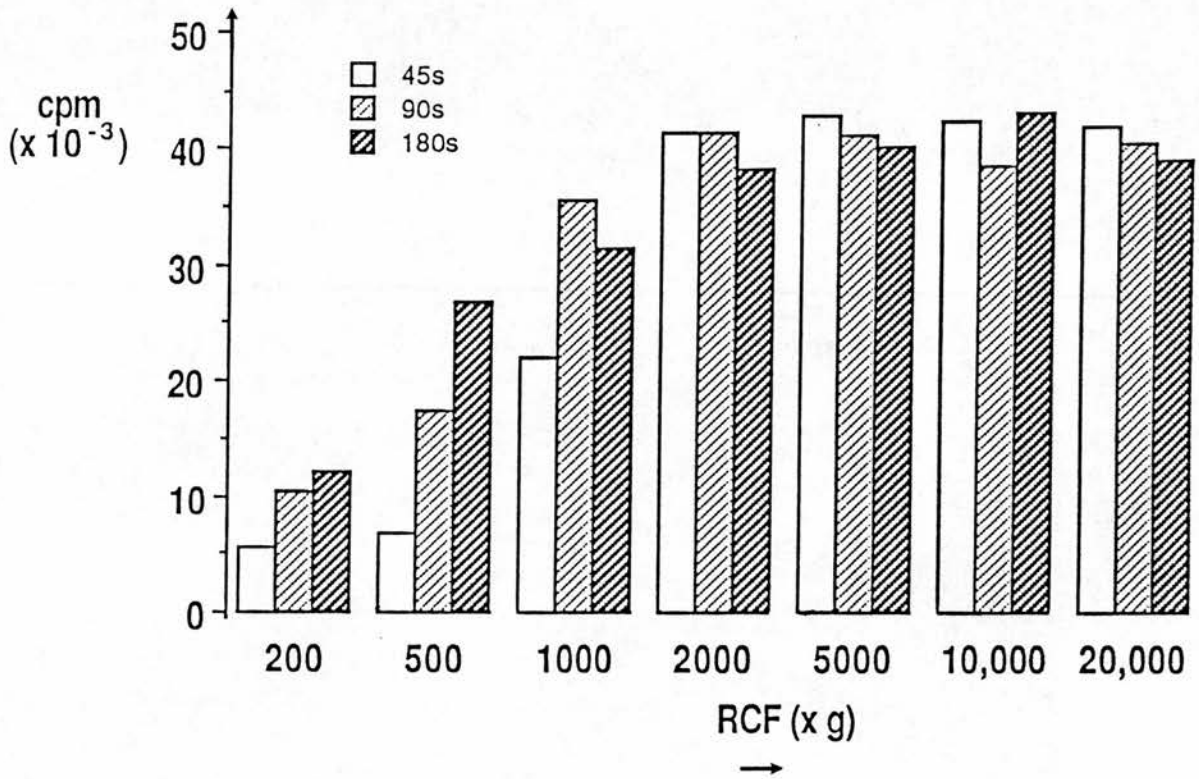


Figure 4.2 Effect of increasing both the duration of centrifugation and the relative centrifugal force (RCF) on the recovery of radioactivity from the platelet pellet.

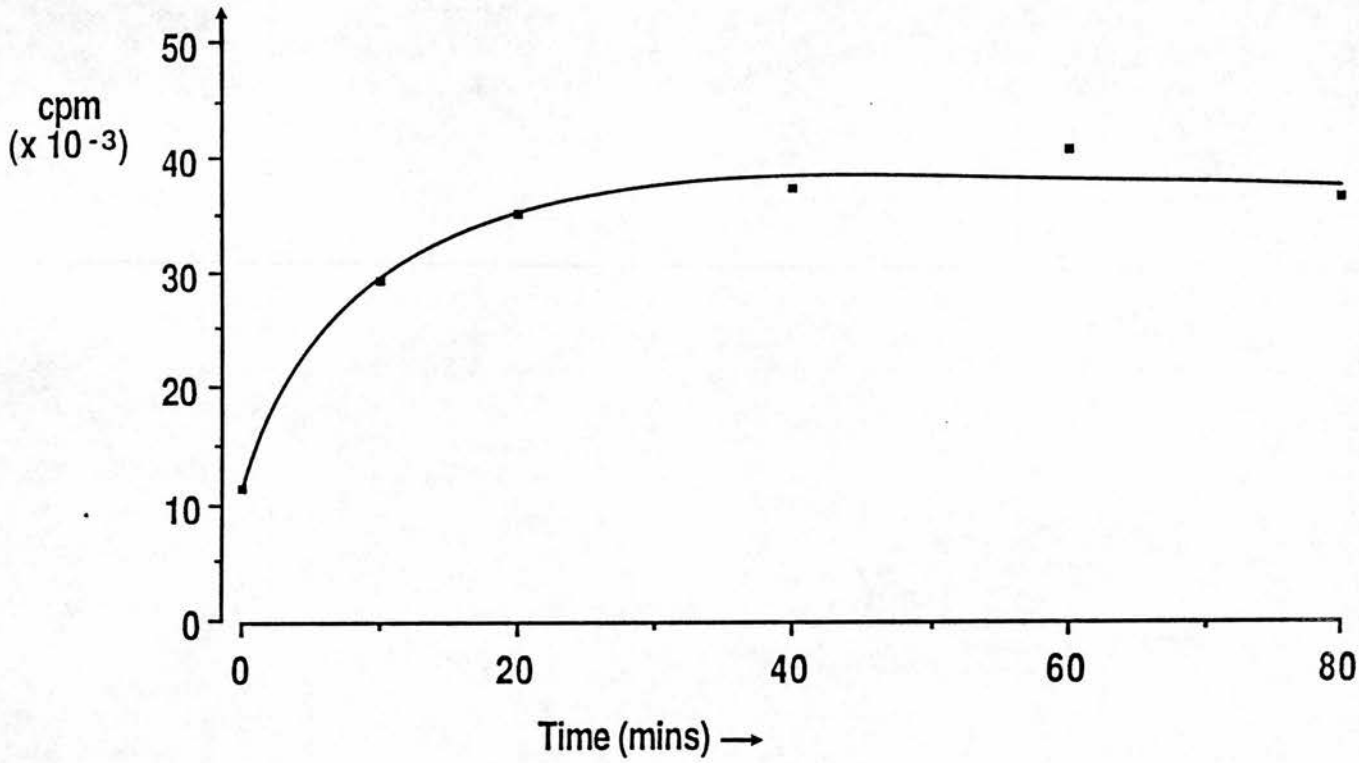
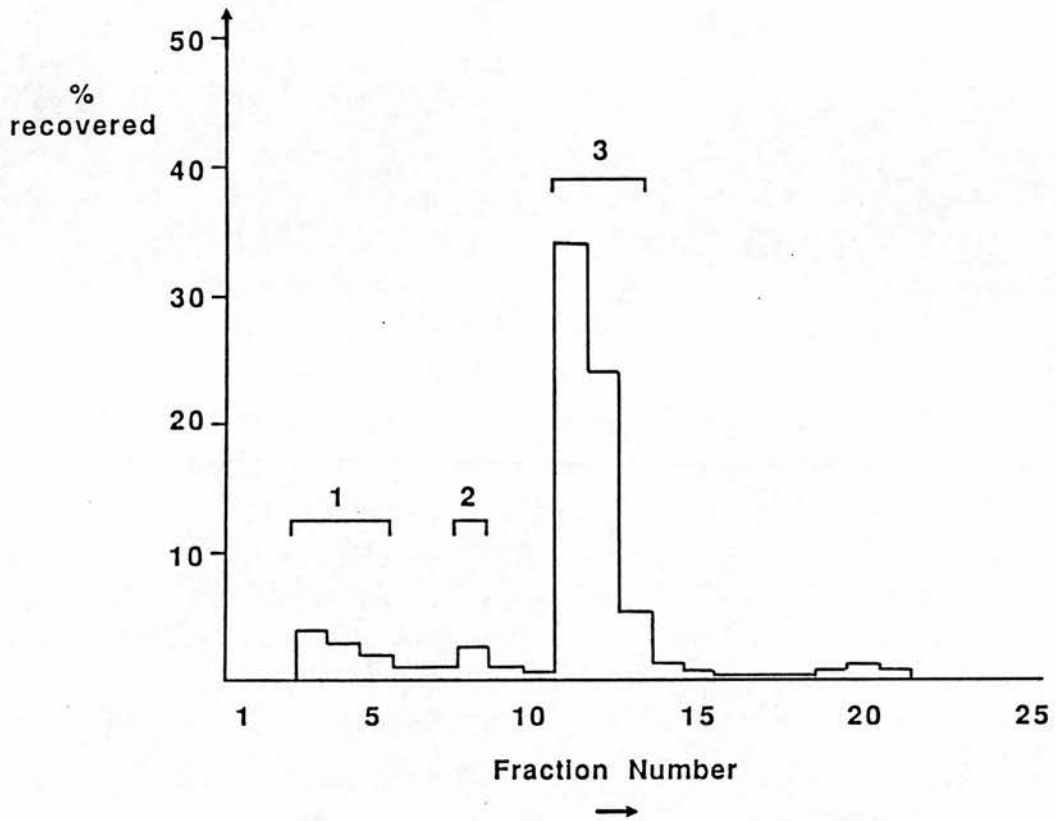


Figure 4.3 Effect of increasing the time allowed for digestion of the platelet pellet produced by centrifugation.

the uptake mechanism, impure [^{3}H]-serotonin was fractionated and used in uptake studies as follows: 200 µl (200 µCi) of stock [^{3}H]-serotonin was injected onto the HPLC (previously calibrated with authentic standard serotonin), and the major peaks of radioactivity which did not correspond to [^{3}H]-serotonin (Figure 4.4) were collected and pooled. Similarly, the peak of [^{3}H]-serotonin was collected, and then diluted in mobile phase to give the same count rate as the pooled impurity peaks. This was to ensure that any observed differences would be due to tracer purity, and not because of different amounts of mobile phase in the incubates. Finally, the purified serotonin tracer and the combined impurities were diluted 1/100 in saline and used immediately in an uptake experiment. The same activity (approximately 2 nCi of [^{3}H]) of purified serotonin tracer or the combined impurities was added per tube for the respective experiments.

For the uptake experiments, standard serotonin was diluted in saline spiked with a low concentration of radioactivity (i.e. the purified serotonin tracer or the combined impurities) to give initial concentrations ranging from approximately 600 nmol/l to 6,000 nmol/l, in dilution steps of 1/3, 1/5, 1/7, and 1/9. PRP was prepared from a normal volunteer, and aliquots (600 µl) pre-incubated in glass tubes for 10 min at 37°C with either normal saline (150 µl) for the active uptake tubes, or chlorimipramine (900 pmol) in normal saline (150 µl) for the passive uptake tubes. After this period, duplicate volumes (2 x 250 µl) of pre-incubated plasma/saline (active uptake), or plasma/chlorimipramine/saline (passive uptake) were added



1 + 2 = IMPURITIES (11.5 % TOTAL)

3 = SEROTONIN PEAK (63.5 % TOTAL)

Figure 4.4 **Profile of radioactivity eluted from the HPLC after injection of impure [^{3}H]-serotonin.**

to aliquots (50 µl) of the non-radioactive serotonin spiked with purified serotonin tracer or the combined impurities, in conical polypropylene microcentrifuge tubes (1.5 ml), which had been pre-warmed for 1 min at 37°C. The tubes were incubated for 1 min each at 37°C, after which time 1 ml of cold (4°C) chlorimipramine in saline (1 µmol/l) was added, the tubes centrifuged as described above, and the supernatant aspirated. The pellets were digested for 1 h at room temperature, and an aliquot from each (200 µl) taken for liquid scintillation counting. To enable accurate timing of the 1 min incubation of PRP at 37°C, each uptake run was staggered, i.e. at each concentration of serotonin being used, the duplicate active and passive uptake tubes were always incubated together, but there was a difference of 15 s between additions to each tube. Active uptake was calculated by subtracting the amount of tracer taken up in the "passive" tube, from the uptake in the "active" tube, and expressing this as a percentage of the total counts added. Final serotonin concentrations were always corrected for the mass of tracer included (approximately 1.25 pmol/tube).

Figure 4.5 shows that there was essentially no active uptake of radioactivity when platelets were incubated with the impurity peak, while there was extensive active uptake of purified tracer into the platelets in the corresponding experiment. Therefore, since the impurity did not contribute to the amount of radioactivity taken up, it was assumed that all the radioactivity actively taken up into the platelets was due to [^{3}H]-serotonin. Hence, for all subsequent uptake studies, unpurified [side chain ^{3}H]-serotonin was

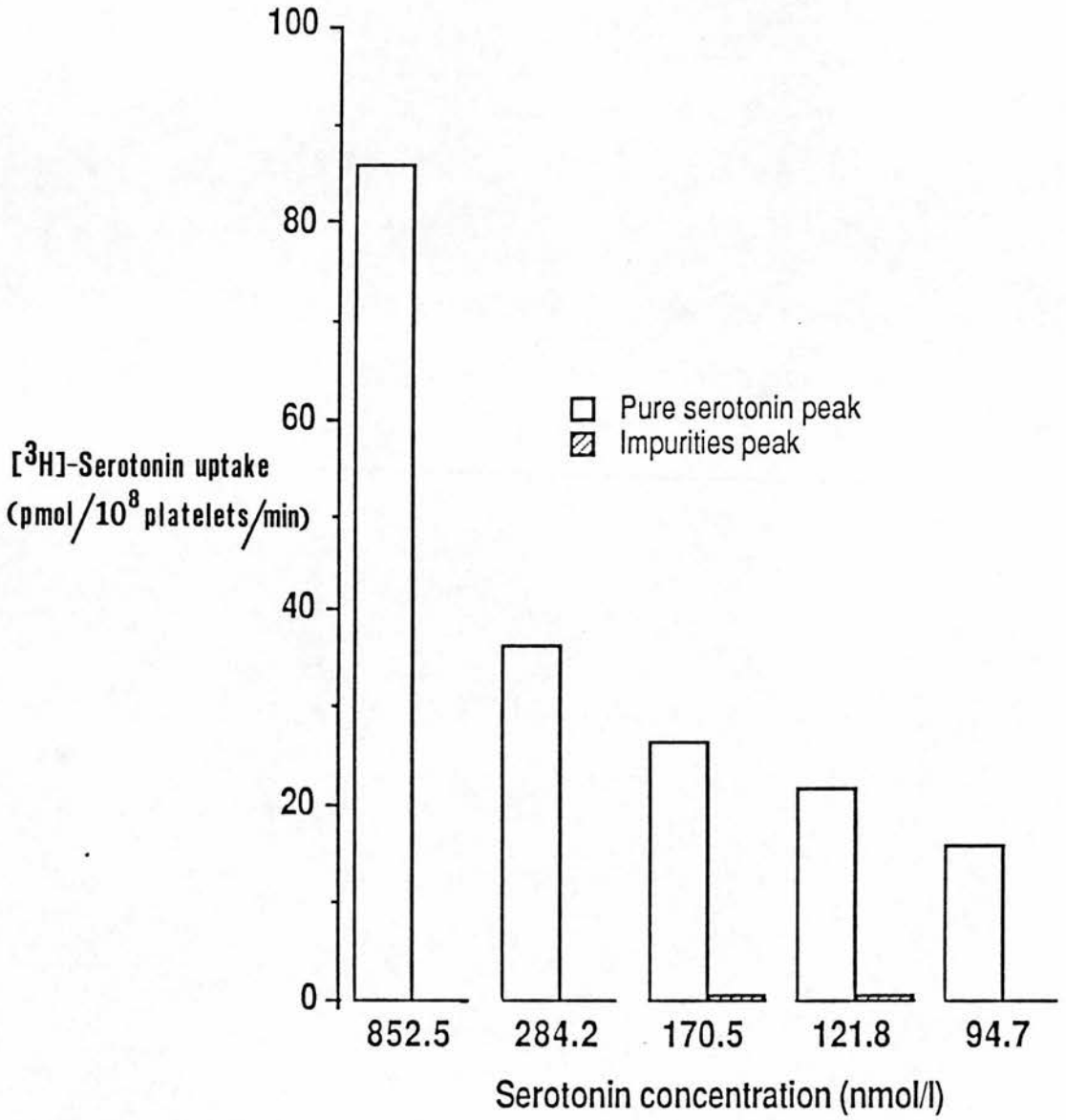


Figure 4.5 Uptake experiments performed with radioactive material collected from either the pooled [^{3}H]-impurity peaks, or from the [^{3}H]-serotonin peak (means of duplicate determinations, values corrected for passive diffusion).

used to spike varying concentrations of non-radioactive serotonin, the range of final concentrations used being approximately 100 - 1,000 nmol/l, in dilution steps of 1/3, 1/5, 1/7, and 1/9. The total counts were always corrected for the purity as determined by HPLC (e.g. if only 75% of the activity of the tracer was associated with the serotonin peak, then uptake was calculated as a percentage of 75% of the total counts). In order to monitor tracer purity, each batch of [^{3}H]-serotonin was analysed by HPLC at intervals of four weeks, and was no longer used for uptake studies once the purity had fallen below 60%.

4.2.5 The Effect of Diluting Platelets on Uptake

For these experiments, Krebs-Henseleit saline (KHS) was prepared as described (Krebs, 1950), but using NaCl to replace the sodium salts of the amino acids originally used (Table 4.2 and 4.3). The KHS was diluted with ACD (the anticoagulant used for blood collection) in the ratio of 9 parts KHS:1 part ACD (the KHS had been gassed with 95% O_2:5% CO_2 for 1 h prior to dilution). Fresh PRP was prepared from a normal volunteer, and uptake studies carried out as described above, either with neat PRP, or PRP diluted 1/5 in KHS/ACD.

The percentage uptake in the diluted samples were significantly lower than those obtained with the undiluted PRP ($p<0.05$, Student's *t* test), but comparison of the results by linear regression analysis (Figure 4.6) showed a good correlation ($r = 0.976$) between uptake after 1 min in the undiluted samples compared with uptake (corrected for platelet number) in samples diluted in KHS/ACD. Thus any

Component	Formula weight	Stock (mmol/l)	Volume added (ml)	Mass added (mmol)	Conc. (mmol/l)	Mass added (mg)	Conc (mg/l)
NaCl	58.4	154	100	15.4	118.5	899.4	6920
KCl	75.6	154	3	0.5	3.6	34.5	265
$CaCl_2$	147.0	110	3	0.3	2.54	48.5	373
KH_2PO_4	136.1	154	1	0.2	1.2	0.02	163
$MgSO_4.7H_2O$	246.5	155	1	0.2	1.2	0.04	294
$NaHCO_3$	84.0	154	21	3.2	24.8	271.4	2087
Glucose	180.2	1 500	1	1.5	11.5	2.7	210

Table 4.2 Composition of The Krebs-Henseleit saline used for diluting platelets (after Krebs, 1950).

Component	Final Concentration (mmol/l)					
	KHS	KHS +ACD	199/EBSS	199/EBSS +ACD	Plasma	Plasma +ACD
Na	143.3	161.4	143.3	161.5	138-148	157-166
K	4.72	4.26	5.36	4.82	3.84-5.12	3.46-4.61
Ca	2.54	2.29	1.81	1.63	2.50-2.74	2.25-2.47
Mg	1.19	1.07	0.81	0.73	0.65-1.05	0.59-1.45
Cl	127.1	114.4	125.2	112.7	101.6-106.6	91.4-95.9
PO_4	1.18	1.06	1.01	0.91	0.65-1.61	0.54-0.95
SO_4	1.19	1.06	0.81	0.73	0.15-0.50	0.14-0.15
HCO_3	24.8	22.4	26.2	23.6	21.3-28.5	19.2-25.7
Glucose	11.54	21.5	5.55	16.1	6.30-8.90	16.8-19.1

Table 4.3 **Comparison of the composition of Krebs-Henseleit saline (KHS), Medium 199 with Earle's balanced salt solution (199/EBSS), and normal human plasma, before and after dilution with acid-citrate-dextrose anticoagulant (ACD).**

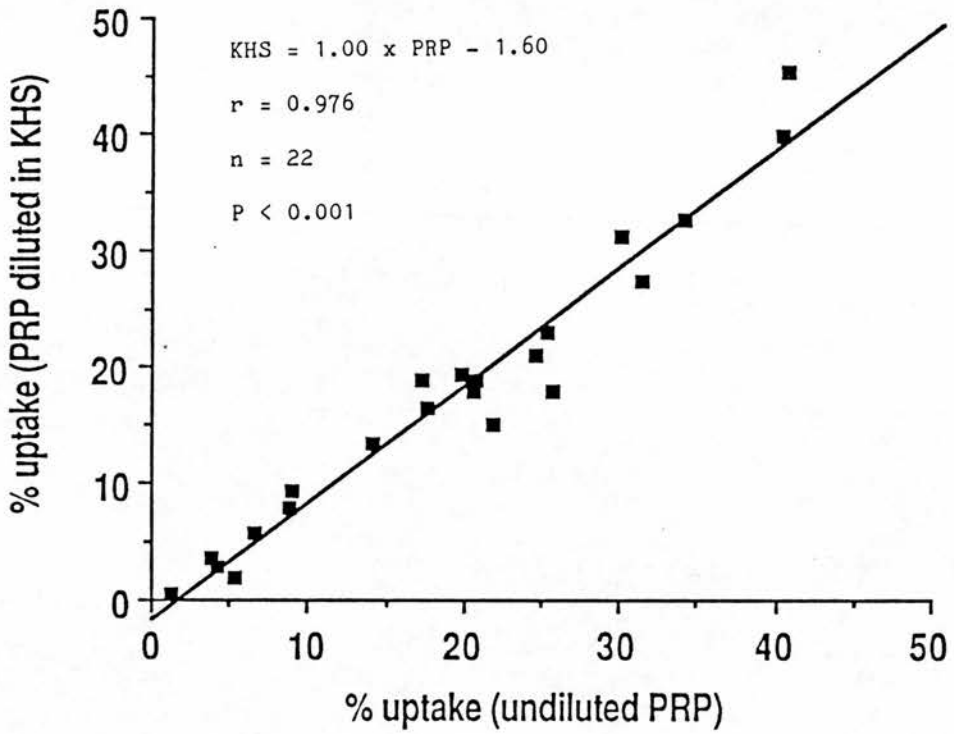


Figure 4.6 Correlation between the uptake of [^{3}H]-serotonin by normal human platelets determined either in undiluted PRP, or in PRP Diluted 1/5 in KHS (means of duplicate determinations, values corrected for passive diffusion).

changes in the extent of uptake seen using undiluted PRP should also be seen using the diluted samples. For all further uptake experiments, PRP was diluted 1/5 in fresh KHS/ACD, which had been gassed with 95% O_2:5% CO_2 for 1 h.

4.2.6 Effect of Storing Diluted PRP at Room Temperature Prior to Uptake

Aliquots (750 µl) of fresh PRP were diluted 1/5 in KHS/ACD, dispensed into glass test tubes, and left uncapped at room temperature for 0, 30, 60, 90, or 120 min prior to a portion (700 µl) being dispensed into clean, glass tubes. These were pre-incubated either with saline, or saline/chlorimipramine (175 µl), followed by incubation in triplicate with [^{3}H]-serotonin/non-radioactive serotonin for 1 min at 37°C as desribed above, except that only one concentration of tracer/cold serotonin was used (approximately 100 nmol/l final concentration). Uptake was halted by the addition of 1 ml ice-cold chlorimipramine in saline (1 µmol/l), the tubes centrifuged, the supernatant aspirated, and the pellet digested.

The results (Figure 4.7) show a significant decrease ($p<0.05$, Student's t test) in the active uptake when diluted PRP was left for >30 min at room temperature, but no significant change in passive diffusion for up to two hours from the start of the experiment. To try and minimise this apparent decrease in function, in all later uptake experiments, PRP or diluted PRP was prepared and stored in stoppered polystyrene tube, with the air above the PRP/diluted PRP replaced by a mixture of 95% O_2:5% CO_2.

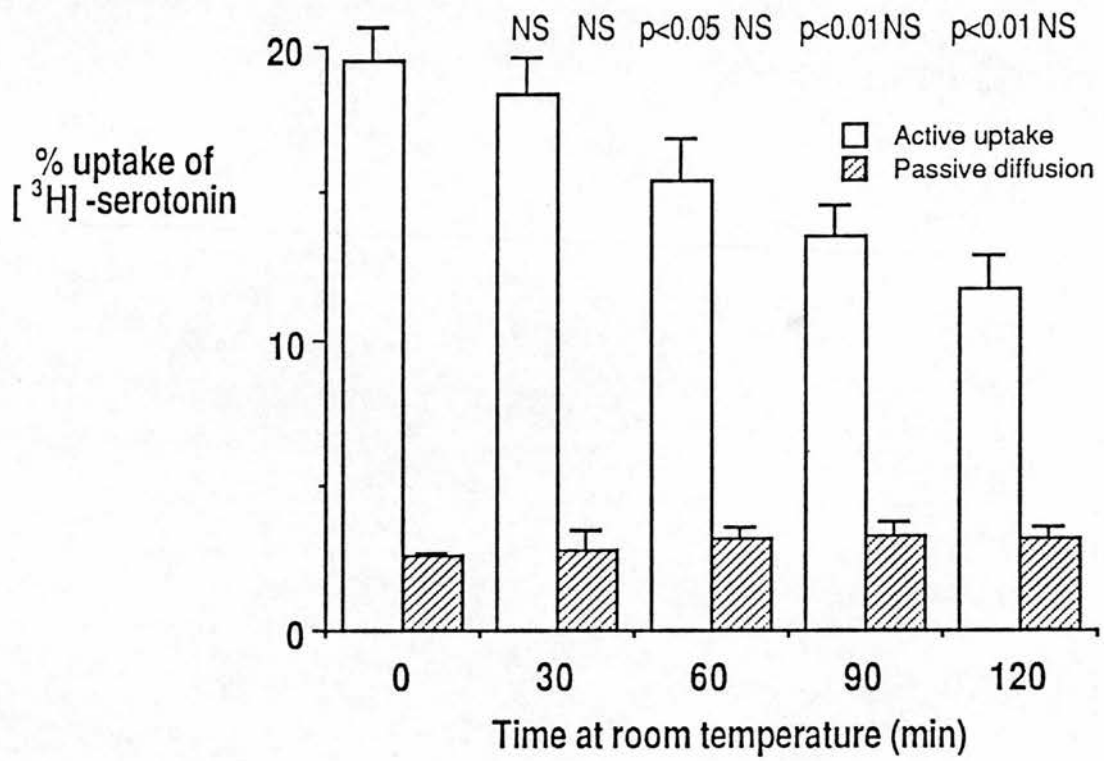


Figure 4.7 Effect of leaving diluted PRP at room temperature and exposed to the atmosphere, on the accumulation of [^{3}H]-serotonin by human platelets (mean + SD of triplicate determinations, active uptake corrected for passive diffusion, p values determined by Student's t test).

4.2.7 Calculation of Maximum Velocity (V_{max}), and Michaelis Constant (K_m) of Uptake of [^{3}H]-serotonin

To calculate the paramaters of uptake, V_{max}, and K_m, a program (Appendix 1) was written to run in Microsoft BASIC on an IBM-PC or compatible microcomputer. The program was based on the direct linear plot, a non-parametric analysis, as described by Eisenthal and Cornish-Bowden (1974), with later modifications (Cornish-Bowden and Eisenthal, 1978). This plot constructs a line from each substrate concentration and its related initial velocity; the points of intersection of these lines represent estimates of V_{max} and K_m. These values are ranked in ascending order, and the median values taken to be the best estimates of the "true" V_{max} and K_m. The computer program was used to calculate the equation of a line of best fit for each uptake study, from a set of Lineweaver-Burk co-ordinates derived from the experimental data (i.e. 1/initial velocity of uptake, $1/v_i$, and 1/serotonin concentration used, 1/S). The data were plotted as a double reciprocal, from the equation

$$\frac{1}{v_i} = \frac{1}{V_{max}} + \frac{K_m}{V_{max} \cdot s}$$

which results in a straight line. The uptake parameters V_{max} and K_m were then calculated from the intercept on the **y**-axis ($1/V_{max}$), and the slope of the line (K_m/V_{max}) respectively.

Figure 4.8 shows a set of uptake data plotted using the revised Eisenthal/Cornish-Bowden method, and Table 4.4 is the calculated V_{max} and K_m for each intersection after re-arranging into ascending order.

Comparison with the results from the computer program (Table 4.4)

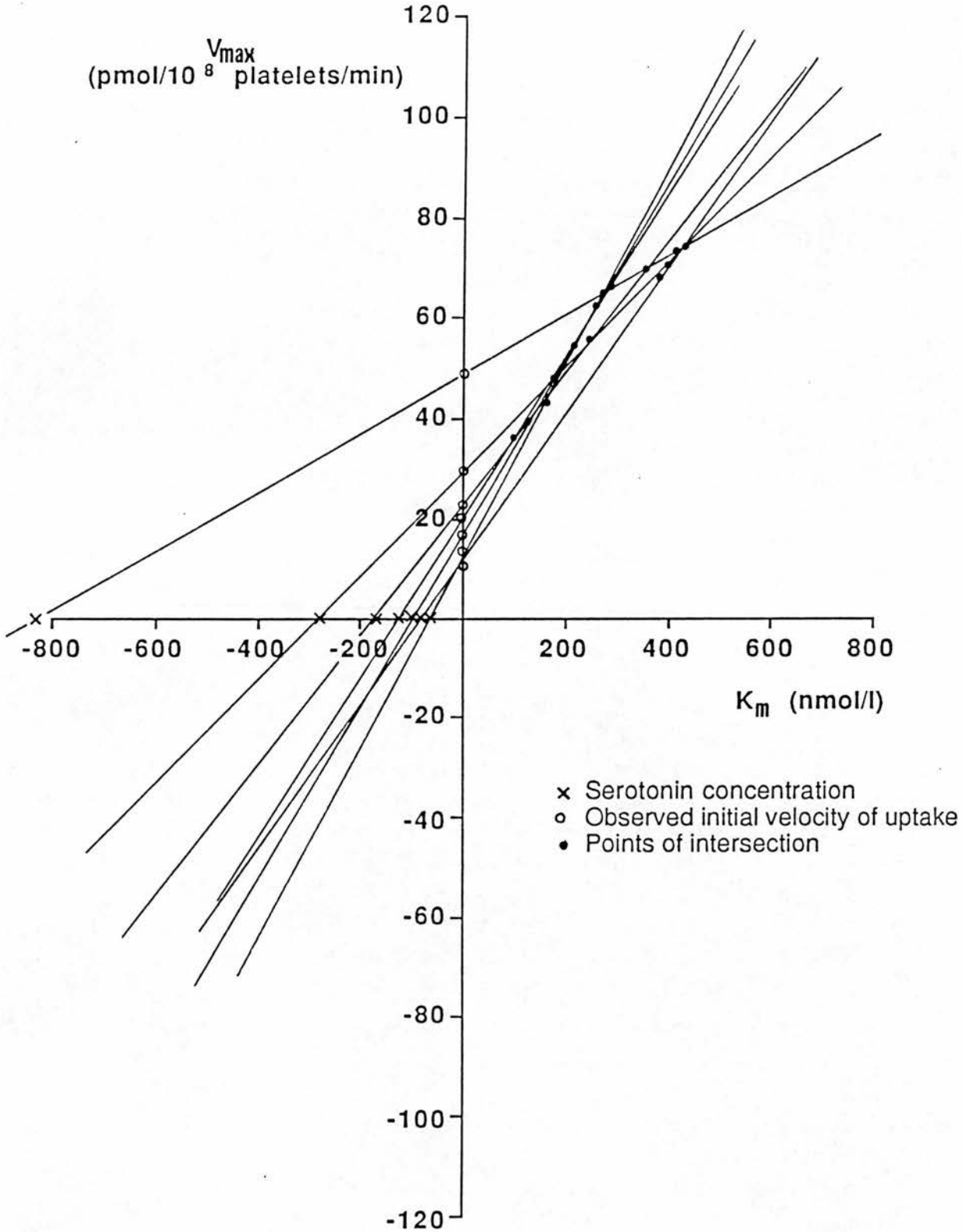


Figure 4.8 Data from a typical uptake study plotted as a direct linear plot (mean of duplicate estimations of V_{max}, corrected for passive diffusion).

Direct Linear Plot		IBM Program	
V_{max} (pmol/10^8 platelets/min	K_m (nmol/l)	Gradient	Intercept
-26.8	7.6	-0.385	0
100.0	34.8	0.281	0
128.6	38.5	0.330	0.902
163.2	42.4	0.372	1.355
174.1	46.4	0.375	1.383
175.4	46.5	0.380	1.440
180.0	47.3	0.381	1.458
220.0	53.5	0.397	1.540
248.2	53.9	0.413	1.560
260.0	61.0	0.432	1.560
275.2	**63.8**	**0.433**	**1.574**
277.2	63.9	0.434	1.829
291.9	64.7	0.450	1.863
363.9	66.6	0.461	2.090
372.1	68.8	0.533	2.103
397.7	69.2	0.575	2.169
415.9	71.8	0.581	2.180
433.3	72.8	0.604	2.337
650.0	105.8	0.615	2.631
-192.0	-16.4	1.000	2.922
-700.0	-90.0	1.000	13.171
Median V_{max} = 63.8		Median V_{max} = 63.5	
Median K_m = 275.2		Median K_m = 275.1	

Table 4.4 **Calculation of parameters of uptake kinetics (V_{max} and K_m) by direct linear plot, or IBM computer program, from an uptake experiment using platelets from a patient with essential hypertension.**

used on the same data shows that both methods give comparable results for the same original set of data.

The Michaelis-Menten parameters V_{max} and K_m for serotonin uptake into platelets from normal volunteers are shown in Table 4.5, together with the intra- and inter-volunteer variation.

4.3 Platelet Aggregation

Platelet aggregation was determined after the method of Born (1962). Briefly, PRP was prepared as previously described, and autologous PPP was obtained by further centrifugation (20 min at 3,025 x g at room temperature) of the red cell fraction which remained after removal of the PRP. The PRP was stored under an atmosphere of 95% O_2:5% CO_2 as described for the [^{3}H]-serotonin uptake studies, and was used within an hour of preparation.
The aggregometer was calibrated by designating the amount of light (wavelength 660 nm) transmitted through the PRP sample as 0%, and the amount of light transmitted through the PPP sample as 100%. To determine the extent of aggregation, an aliquot of fresh PRP (990 µl) was dispensed into a disposable clear polystyrene tube, and pre-incubated for 5 min at 37°C, whilst being stirred (1,100 rpm) by a small (1 x 3 mm), polypropylene-coated magnetic stirring-rod. Aggregation was induced by the addition of ADP in 199/ACD (10 µl, pH 7.4), to give final concentrations of up to 4 µmol ADP/l. The extent of aggregation (%) was expressed as the maximum increase in light transmission obtained with the aggregating agent, relative to the reference points established with PRP (0%) and PPP (100%). When

Uptake	$V_{max} \pm SD$ (pmol/10^8 platelets/min)	$K_m \pm SD$ (nmol/l)	n
Intra-individual	120.0 ± 47.6	778.1 ± 384.2	6
% CV	39.7	49.4	
Inter-individual	133.6 ± 87.0	570.3 ± 207.5	8
% CV	65.0	36.4	

Table 4.5 Intra- and Inter-individual variation of [^{3}H]-serotonin uptake parameters in normal male volunteers.

the effect of a drug was being investigated, the procedure was as described above, except for the following changes:

1) the volume of PRP used was 890 µl,
2) the pre-incubation time was 3 min,
3) after pre-incubation, 50 µl of 199/ACD (pH 7.4) was added, followed by a further 1 min incubation, and
4) either 199/ACD (50 µl, pH 7.4), or the drug diluted in 199/ACD (50 µl) was added, incubated for 1 min, after which aggregation was induced by addition of ADP as above.

In each case, aggregation was allowed to proceed for 4-5 min, and, where aggregation was carried out on more than two samples, tubes for pre-incubation were staggered at intervals of 3-4 min, in a manner similar to that employed for the [^{3}H]-serotonin uptake experiments.

The variation seen in induced aggregation in the same volunteer within run, or between runs is shown in Table 4.6. Figure 4.9 shows typical profiles obtained when platelets from a normal volunteer are aggregated by ADP.

4.4 Discussion

Blood samples were collected from subjects at approximately the same time of day to avoid any variations which may be due to circadian fluctuations of uptake, but generally, no special precautions were taken to ensure that subjects had fasted prior to venesection. Apparent rhythms of uptake have been reported in normals (Arora, et al., 1984) and in depressed patients (Rausch, et al, 1982). Also, nyctohemeral variations in serotonin

Parameter	% Aggregation (± SD)	% CV	n
Intra-run	41.6 ± 1.8	4.3	3
Inter-run	48.2 ± 5.4	11.2	5

Table 4.6 **Intra- and Inter-run variation in percentage aggregation induced by ADP (4 µmol/l) in PRP from a normal volunteer.**

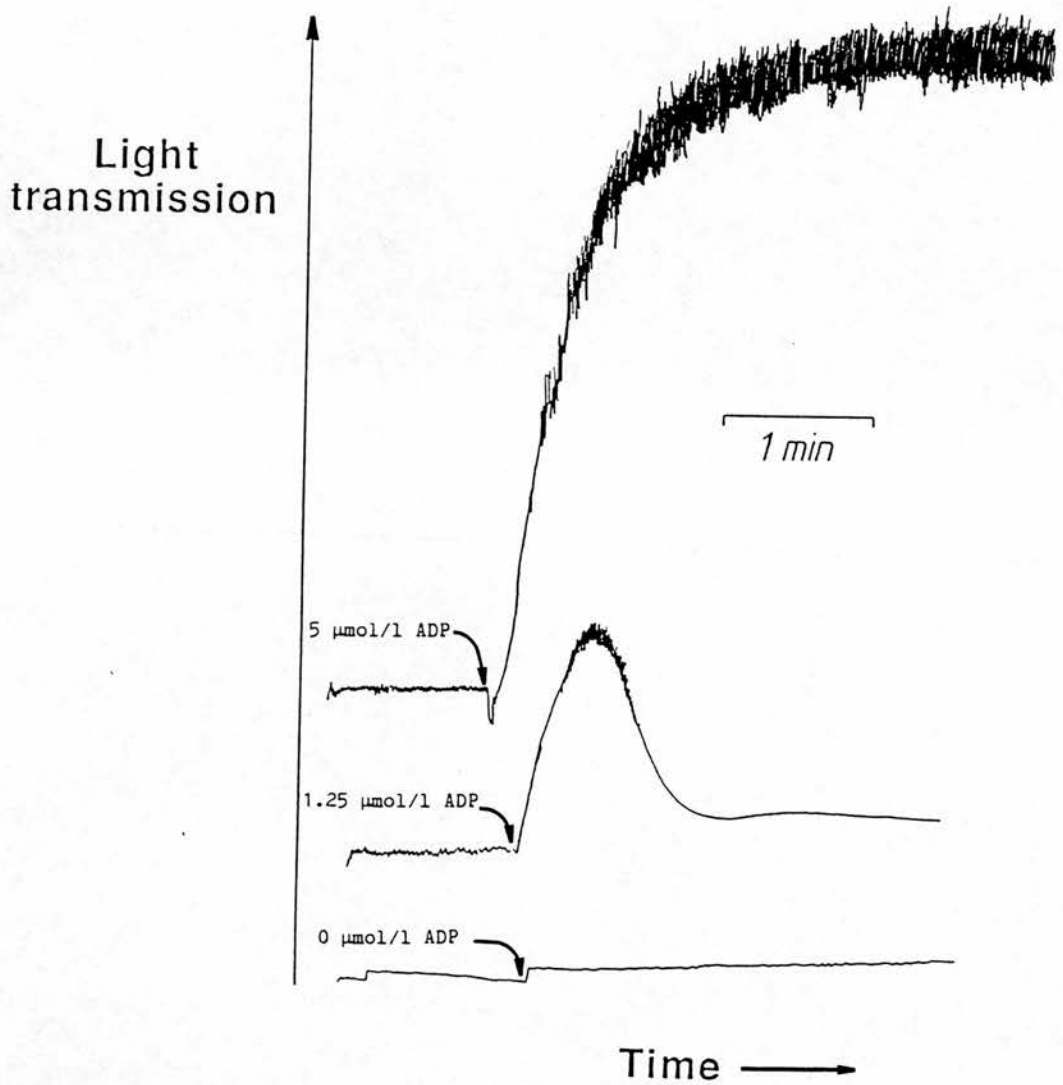


Figure 4.9 Profiles of the ADP-induced aggregation of platelets from a normal volunteer.

concentration have been described in the pineal gland of the rabbit, with levels being highest during the day (Brainard, et al., 1984); rat serum serotonin has also been shown to fluctuate over a 24 h period (Ho, et al., 1985), with maximal values occurring during the light period. Whether these observed increases in serotonin concentration are due to intrinsic rhythmic changes in the rate of uptake of the indoleamine into platelets remains to be clarified, but it has been suggested that the frequency of feeding is a stronger entrainer of the rhythm of serum serotonin in the rat than the light/dark cycle (Ho, et al., 1985). A post-prandial increase in blood serotonin has been reported in normal human volunteers (Kellum and Jaffe, 1976); this effect may be due to release of serotonin from the gut enterochromaffin cells due to direct stimulation of the intestinal mucosa (Biber, et al., 1974), or an increase in synthesis of serotonin due to increased availability of the serotonin precursor, tryptophan, from ingested food (Feltkamp, et al., 1984), since it has been suggested that the concentration of tryptophan is the rate-limiting factor in serotonin synthesis (Lovenberg, et al., 1967; Friedman, et al., 1972).

Slow-speed centrifugation of whole blood to prepare PRP has been criticised since it is possible that the most dense platelets will be spun down and lost from the PRP (Healy and Eagen, 1984). The similarity between the profiles of platelet volume obtained from citrated whole blood and from PRP prepared as described suggests that the size distribution of platelets harvested from PRP as described in this thesis is similar to that which exists in whole blood.

Platelets isolated from whole blood for function tests should be representative of the original total platelet population, otherwise it would not be valid for *in vitro* observations to be extrapolated to the *in vivo* situation. Although detailed investigation of this was beyond the scope of this thesis, it should be emphasised that there is much debate in the literature about the relationship between platelet size, density, age, and function in PRP compared with whole blood: Mezzano, *et al.*, (1982) compared platelets recovered from PRP (prepared by differential centrifugation of whole blood), with the population of platelets trapped in the layer of packed red cells. These workers found that 7-43% of platelets in whole blood were trapped in the red cell layer, but both populations of platelets had the same mean volume, size distribution, and similar life-spans, as determined by [^{51}Cr] labelling. The assumption here was that ageing platelets would show a decrease in size and/or an increase in density, and, if the most dense platelets were being trapped by the red cells, there would therefore be an increase in radioactivity recovered from the red cell layer as the population aged.

This was apparently contradicted by a later study (Mezzano, *et al.*, 1984), in which [^{51}Cr] labelled low density human platelets showed a decrease in specific activity compared with a high density sub-population over a period of time, suggesting that newly-formed platelets were low-density, effectively "diluting out" the original labelled low-density cohorts, which would progressivly increase in density as they aged, thus apparently maintaining the specific activity of that high-density sub-population.

Martin, et al., (1983) found virtually no difference in density between platelet sub-populations, and suggested that density is determined at the time of thrombocytopoeisis rather than by ageing; others (Thompson, et al., 1984) showed the same size distribution both in young (2 days post-infusion) and old (9 days post-infusion) [^{75}Se]-methionine-labelled baboon platelets, suggesting that platelet size is age-independant. More recently, (Savage, et al., 1986) showed that there was no difference in the in vivo survival time of either low or high density [^{111}In]-labelled baboon platelets; the latter workers suggest that the great controversy in the literature about the relationship between platelet size, volume, density, and functionality may partly reflect the different methodologies employed. Similar results had been found earlier by Healy and Eagen (1984), who demonstrated that mean platelet volume and distribution width could be affected by choice of anticoagulant, or the method of preparation of PRP, with increasing g forces progressively depleting PRP of the larger platelets.

In summary, it has been assumed in this thesis that platelets harvested by low-speed centrifugation are a fair representation of the total platelet population in whole blood, but it could be argued that the distribution of platelets seen in whole blood ex vivo is a misrepresentation of the in vivo situation, since it has been shown that in the intact rabbit mesenteric artery, the red blood cells flow predominantly in the centre of the vessel, whereas platelets are distributed peripherally around the vessel walls (Tangelder, et al., 1985). The net result is that there is a localised 30% increase

in platelet concentration at the vessel walls compared with that in isolated whole blood.

Visual estimation of platelet numbers by counting in a haemocytometer is a common technique, but it is time consuming, and requires a degree of skill and practice. Furthermore, unless strict precautions are taken to avoid contamination by extrinsic particulate matter (e.g. Born and Hume, 1967), falsely elevated counts will result. For the visual estimations carried out in this thesis, the platelets were not allowed to settle out in the haemocytometer, but an attempt was made to include platelets distributed in different planes by focusing up and down on the sample. Although this enabled a rapid count to be made, it may also account for the poor precision of the visual method. The higher count by the visual method is almost certainly due to inclusion of extrinsic particulate material in the count.

Centrifugal separation of the platelets from the incubation medium after uptake was chosen, since this was rapid, and could easily be performed at 4°C. Isolation of platelets by rapid filtration of the incubate through a small-pore membrane (typically 0.45 μm or less), followed by washing with cold buffer has been used by several other groups to separate the bound and free fractions of [^{3}H]-serotonin (see Table 4.6). This method suffers from the disadvantage that bound tracer may be "washed out" into the filtrate from platelets which lyse due to the washing procedure, and is consequently misclassified as free (Tuomisto, 1974). A similar criticism has been made in a study of platelet [^{3}H]-imipramine binding sites, in which

Platelet Count	Dilution	Incubation Time	Passive Diffusion	Separation	Data Analysis	V_{max} (pmol/10^8 platelets/min)	K_m (μmol/l)	n	Reference
Haemo-cytometer	1/5 (Krebs)	<10 min	4°C	Centri-fugation	Lineweaver-Burk	320	0.5	-	Lemmer, et al., 1977
Haemo-cytometer	None	2 min	4°C	Filtration	Lineweaver-Burk	2.0	0.11	6	Ahtee, et al., 1981
Coulter Counter	1/3 (Krebs)	2 min	4°C/1 μmol/l Chlor-imipramine	Centri-fugation	Woolf plot	107	0.45	8	Arora and Meltzer, 1981
Haemo-cytometer	None	<2 min	4°C	Centri-fugation	Eadie-Hofstee	140	1.20	25	Malmgren, 1981
-	None	2 min	2°C	Centri-fugation	Lineweaver-Burk	32.8	0.51	18	Carroll, et al., 1982
Haemo-cytometer	None	2 min	4°C	Centri-fugation	Lineweaver-Burk	163.9	0.29	15	Kaplan and Mann, 1982
-	Tyrodes	1 min	-	Filtration	-	74.1	0.12	15	Launay, et al., 1982
Haemo-cytometer	None	30 s	1 μmol/l Chlor-imipramine	Filtration	Lineweaver-Burk	41.7	0.7	17	Kamal, et al., 1984a
Coulter Counter	None	30 s	4°C	Filtration	-	23.1	0.57	4	Poirier, et al., 1984
-	None	2 min	Desipramine	Filtration	Iterative	131	0.7	8	Suranyi-Cadotte, et al., 1985

Table 4.7 Conditions used by other groups to study the uptake of serotonin by human platelets

either equilibrium dialysis or vacuum filtration was used to separate the free radioisotope from the bound fraction (Lui, et al., 1984).

To date, there is no evidence in the literature that the effect of tracer purity on the uptake of [^{3}H]-serotonin into platelets has been critically evaluated. In light of the results obtained in this thesis, this is surprising, since the purity of the stock tracer decreased once the sealed vial had been opened, even though the tracer was stored refrigerated as recommended by the manufacturers. Thus, if no correction factor is applied, then as the purity of the tracer decreases, the apparent uptake of serotonin will also decrease. This may partly explain the variations in magnitude of the uptake parameters V_{max} and K_m seen in platelets from normal subjects (Table 4.5). Furthermore, if experiments are badly designed (e.g. control group studied before experimental group), significant differences may be observed due to poor methodology, rather than any real biological difference.

The nature of the impure peaks was not investigated; it is possible that some of the impurity was tritiated water, since this highly polar molecule would not be retained by the hydrophobic column and would elute in the void volume of the system (approximately 2 ml); such a peak is seen in Figure 4.4. It is also possible that some of the impurity is an oxidised derivative of [^{3}H]-serotonin, since the peak appeared after the tracer had been exposed to the atmosphere.

The passive diffusion of [^{3}H]-serotonin into platelets was measured at 37°C using 1 μmol/l chlorimipramine to block active

uptake (Kamal, et al., 1984a) rather than incubating a parallel set of tubes at 4°C. This was intended to reflect more accurately diffusion at 37°C, since the rate of passive diffusion is known to increase with increasing temperature (Arora and Meltzer, 1981). Chlorimipramine was used since this is approximately 5 times more potent than imipramine at blocking uptake, (Todrick and Tait, 1969).

PRP stored in an open container rapidly loses CO_2 to the atmosphere (Terres, et al., 1986), and becomes alkaline (Day, et al., 1975). This rise in pH is accompanied by an apparent change in function, notably a decrease in V_{max} (Lemmer, et al., 1977), increased responsiveness to aggregating agents (Tang and Frojmovic, 1977), and an increase in the rate of shape change (Malmgren, et al., 1985). Of the methods used to try and minimise the loss of CO_2, one of the most common is to seal the PRP in a tube, after replacing the air above the PRP with a mixture of 95% air/5% CO_2 or similar; use of this method maintained the pH of citrated PRP at approximately 7.3 for up to 3 h at 37°C (Tang and Frojmovic, 1977), with no increase in function as measured by the aggregation response: Tang and Frojmovic (1977) suggested that the increase in PRP pH may somehow lead to an increased mobilisation of calcium ions, which results in an enhanced aggregation response. For the studies in this thesis, the PRP or diluted PRP used for function studies was stored under 95% O_2/5% CO_2 and at room temperature, in order to minimise alterations in platelet function (Lemmer, et al., 1977; Tang and Frojmovic, 1977).

In the uptake studies, since only seven concentrations of [^{3}H]-serotonin/cold serotonin were used for each uptake, the time interval

between the start of the experiment and the last tube being pre-incubated would be 30 min: this is calculated on the basis of a 10 min pre-incubation period for each concentration point, with tubes set up for pre-incubation at intervals of 5 min. The time taken for the uptake, centrifugation, and aspiration (which includes pipetting, loading/unloading centrifuge, the acceleration or deceleration etc.), can be estimated by allowing 2 min for the uptake procedure overall, 1 min 30 s for centrifugation, and 30 s for aspiration, total time taken = 4 min, which leaves an interval of 1 min before the start of the next incubation period. Thus even if the diluted PRP was left exposed to the atmosphere, it is unlikely that significant decreases in uptake would be seen.

There are several reasons why PRP was diluted prior to being used in uptake studies: firstly, it enables uptakes to be performed over a wide range of serotonin concentrations using a minimum volume of PRP, and this was expected to result in a more accurate determination of V_{max} and K_m. Secondly, dilution slows down the rate of uptake on a "per tube" basis. This was necessary, since otherwise a large proportion of free substrate (>25%) may be taken up by the platelets after incubation for 1 min at 37°C (Malmgren, 1981); in such a situation, it would not be valid to apply Michaelis-Menten kinetics to the uptake process (Tuomisto, 1974), since a fundamental assumption in the derivation of the Michaelis-Menten equation is that there is no change in substrate concentration at initial velocity. The uptake of serotonin by human platelets is linear with respect to time for up to six minutes after the start of incubation (Arora and

Meltzer, 1984), and the rate of uptake during the first minute is taken to be the same as the initial velocity. This may explain the observed differences in uptake between the diluted and undiluted PRP, since in the undiluted samples, there will be a rapid substrate depletion over the incubation period; thus in the two sets of tubes, it is probable that the uptake process will be following two different types of kinetics. As a method of reducing the rate of [^{3}H]-serotonin uptake per tube, dilution of PRP was more practical than increasing the masses of substrate used, or decreasing the incubation time to <1 min (Tuomisto, 1974; Malmgren, 1981). Finally, it was hoped that dilution of PRP in a bicarbonate buffer (i.e. KHS) well-saturated with CO_2 would minimise the functional changes associated with an increase in pH due to loss of CO_2 to the atmosphere.

In serotonin uptake studies, it is common practice to calculate the V_{max} and K_m of the reaction from a straight line fitted by least-squares linear regression to a Lineweaver-Burk plot of the experimental data (e.g. Kamal, et al., 1984a; Lee and Chan, 1984; Guicheny, et al., 1985). This technique has been criticised, since unless proper attention is given to "weighting" of the data points with the best precision, the least squares plot is unsatisfactory (Nimmo and Atkins, 1979; Atkins and Nimmo, 1980).

Furthermore, in the Lineweaver-Burk plot, the initial velocity (v_i) is plotted as:

$$\frac{1}{v_i} = \frac{1}{V_{max}} + \frac{K_m}{V_{max} \cdot s}$$

where K_m is the Michaelis constant, v_i is initial velocity, V_{max} is maximum velocity, and s is substrate concentration; the result of this is that undue emphasis is given to the smallest v_i, which will probably contain the largest errors (Dowds and Riggs, 1965). The revised direct linear plot (Cornish-Bowden and Eisenthal, 1978) is a distribution-free method of fitting a line to a set of points, and is thought to be more robust and reliable than the Lineweaver-Burk plot with linear regression (Dowd and Riggs, 1965; Nimmo and Atkins, 1979; Atkins and Nimmo, 1980). The revised version of the plot is equivalent to fitting a line to data for $1/v_i$ and $1/_s$ (Nimmo and Atkins, 1974), which was the method used in this thesis. Finally, Nimmo and Atkins (1979) concluded that the direct linear plot

> "...in general, appears to be superior to that of least squares... Consequently, we believe the revised version of the plot should become the standard method for estimating Michaelis constants."

In essence, the computer program calculates the equation of the line between each data point, and every other data point; it has been shown that for n co-ordinates, there are 0.5 x n x (n-1) possible lines (Nimmo and Atkins, 1979). The gradients and intercepts of these lines are then calculated algebraically (statements 270-340), sorted into ascending order (statements 520-580), and the median values

taken as representing the line of best fit for those data (statements 740-860). In the revised plot (and as used by the computer program), negative intercepts on the y-axis (i.e. negative $1/V_{max}$) are set to zero (statement 330), and their corresponding gradients (K_m/V_{max}) set to 1 (statement 330), thus making V_{max} and K_m very large, as recommended in the modified original method (Cornish-Bowden and Eisenthal 1978).

Platelet aggregation in vitro is a common test of platelet function, and the methodology is well-established. However, as with serotonin uptake studies, attention must be paid to the possibility of spurious results resulting from technical rather than biological differences. For example, after preparation, the PRP was stored at room temperature sealed under an atmosphere of 5% CO_2, and aggregation studies were completed within an hour of preparing the PRP. The PRP was not diluted down with autologous PPP to give constant counts for each experiment for two reasons: firstly, PPP is known to contain "free" serotonin, the concentration of which may depend on the degree of trauma during venesection, or the subsequent processing of the blood/PRP. Serotonin itself can induce, potentiate, or inhibit platelet aggregation (Baumgartner and Born, 1968), and it was felt that dilution with PPP which may contain varying amounts of serotonin between experiments would be introducing an unacceptable variable. Secondly, variations in the platelet count do not appear to affect the aggregation response, unless the concentration is $<75 \times 10^9$ platelets/l, at which point there is a significant decrease in the extent of aggregation (Roper, et al.,

1974; Levine, 1976).

Adenosine diphosphate (ADP) was used to induce aggregation, since ADP may be released from injured blood vessels *in vivo* (Born, 1962), or from damaged red cells (Born, 1962; Born, *et al.*, 1976; Born, 1985). Therefore it was anticipated that the platelet response to ADP *in vitro* would best reflect events which may be occurring *in vivo*.

Chapter 5

Serotonin, Angiotensin II, and Platelet Aggregation

5.1 Introduction

The steroid hormone aldosterone is known to be the major inhibitor of natriuresis in higher mammals (see Fraser and Padfield, 1985). The increased production of aldosterone seen during sodium-restriction results from increased stimulation of the zona glomerulosa cells of the adrenal cortex by angiotensin II (AII), the circulating levels of which increase during sodium deprivation; conversely, during sodium loading, AII synthesis is reduced, which in turn results in diminished aldosterone production (Figure 5.1). Serotonin has also been shown to stimulate aldosterone production from isolated zona glomerulosa cells *in vitro* (Haning, *et al.*, 1970; Williams, *et al.*, 1984), and elevated plasma levels of aldosterone in humans have been demonstrated after infusions of serotonin in man (Mantero, *et al.*, 1982). However, the relationship between serotonin and sodium status *in vivo* remains to be clarified. It has been demonstrated that AII has a stimulatory effect *in vitro* on the activity of tryptophan hydroxylase (possibly through its metabolite AIII (Nahmod, *et al.*, 1978)), which is the rate limiting step in serotonin biosynthesis; thus it may be argued that increased levels of AII should lead to increased serotonin synthesis.

Angiotensin II is known to affect the response of platelets to aggregating agents *in vitro*: high concentrations (10^{-7} mol/l) of AII inhibited the extent and velocity of adrenaline-induced platelet aggregation (Ding, *et al.*, 1985b), whereas low concentrations (10^{-9}-10^{-11} mol/l) of AII potentiated the aggregation response (Poplawski, 1970; Ding, *et al.*, 1985b). Furthermore, specific AII receptors

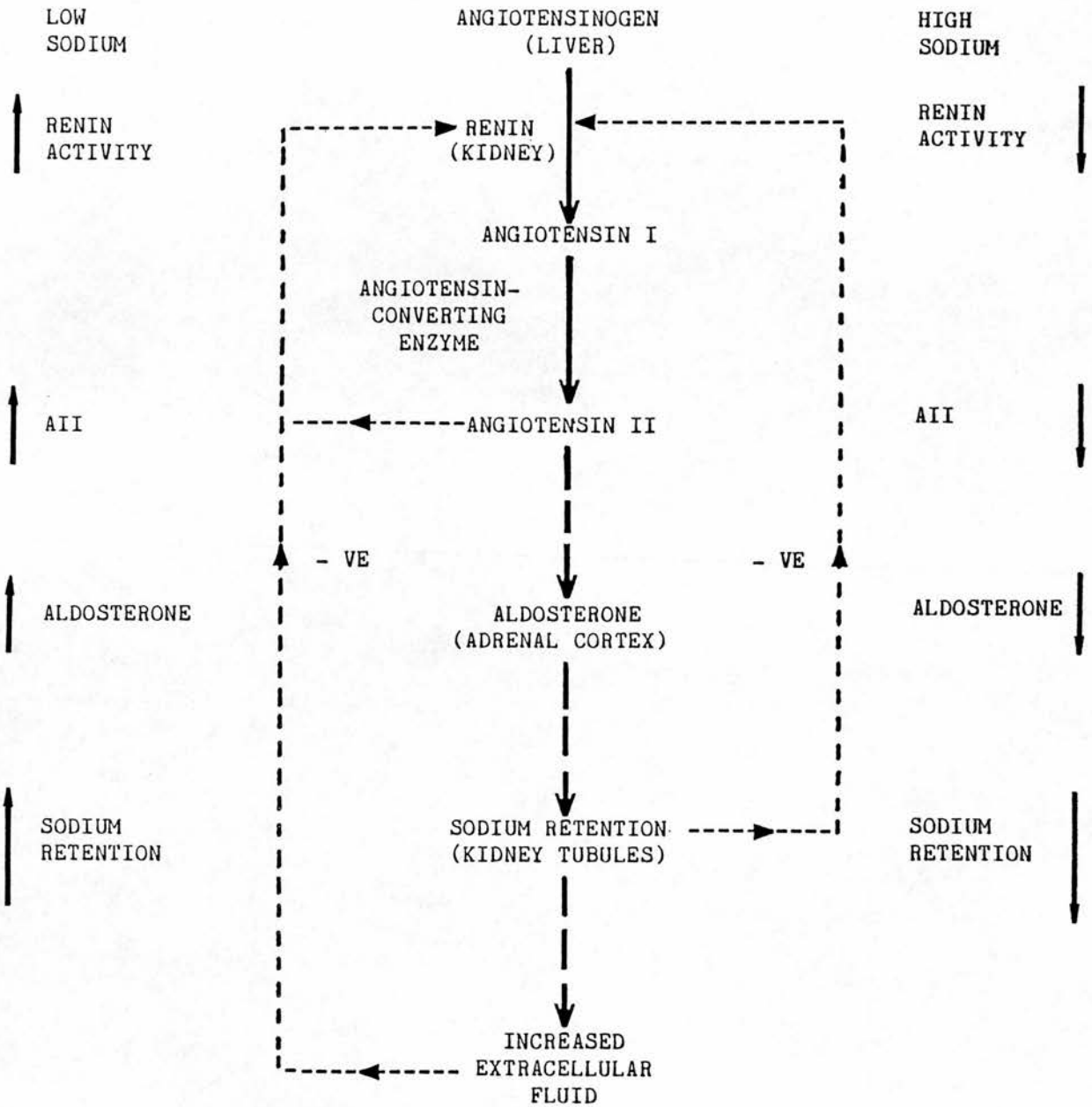


Figure 5.1 The relationship between sodium status and the renin-angiotensin-aldosterone system

——→ = conversion

— — → = action at target site

--------→ = negative feedback.

have been shown to exist on human platelets, and the number of these receptors was significantly reduced in normal human subjects during a low salt diet (Ding, et al., 1985a). Serotonin also affects in vitro platelet aggregation in a pattern similar to that seen with AII: high doses of serotonin inhibit ADP-induced platelet aggregation, whereas low doses cause potentiation of the aggregation response (Baumgartner and Born, 1968). Receptors for serotonin have been shown to exist on human platelets (Peters and Grahame-Smith, 1980), but it is not yet known if these are affected by changes in dietary sodium intake.

This chapter describes experiments which were carried out to investigate the effect of variations in sodium intake on serum serotonin levels in rats, followed by further work in normal human subjects.

5.2 Effect of Different Sodium Diets on Rat Serum Serotonin

Experiments were performed using normal female Wistar rats from the Animal Unit, Western General Hospital. The rats (weights 175 - 250 g) were housed in the Animal Unit at a constant temperature of 19-21°C, with a day:night cycle of 12 h light:12 h dark. Three separate experiments were performed, each with four groups of five/six rats, maintained on either a standard diet (CRX rat chow), low, normal or high sodium diet (Table 5.1) for 14 days. Blood was collected on the morning of day 15 by cardiac puncture (following ether anaesthesia of the rats), and serum prepared as described above (3.2.6b). The serum was initially stored at -20°C, and was assayed for serotonin by HPLC within a week of collection. The serum was assayed for aldosterone using a direct RIA (Al-Dujaili and Edwards,

Diet	Low Na^+	Normal Na^+	High Na^+
Wholemeal flour (g)	148.5	148.5	148.5
*Na (mg)	4.5	4.5	4.5
*K (mg)	534.6	534.6	534.6
*Ca (mg)	52.0	52.0	52.0
Calcium carbonate (g)	1.5	0.75	0.75
Sodium chloride (g)	0.0	0.75	0.75
Distilled water	Ad lib.	Ad lib.	Nil
Normal saline	Nil	Nil	Ad lib.

Table 5.1 Daily dietary allowance for rats on varied sodium intake.

(* = from wholemeal flour)

1981) by the staff of the routine RIA laboratory, Department of Medicine, Western General Hospital, Edinburgh. Angiotensin II levels were not measured, since the assay required approximately 5 ml of plasma, and only 4-5 ml of whole blood could be obtained from each rat. Results were analysed by the Student's t test, with $p<0.05$ being taken to represent a significant difference.

There was a significant ($p<0.01$) difference in serum serotonin only when comparing rats on a low sodium diet with rats on a normal sodium diet (Figure 5.2). The expected trends in serum aldosterone levels were seen after the different sodium diets (Table 5.2). The rats on the low sodium diet had significantly higher ($p<0.001$) serum aldosterone levels than those on the standard diet, or normal or high sodium intakes. Aldosterone levels in rats on high sodium intake were not significantly depressed compared with the rats on the normal sodium diet, but were lower ($p<0.02$) when compared with rats on the standard Animal Unit diet. There was however, no significant difference in serum aldosterone levels between the standard diet and the normal sodium diets.

5.3 Effect of Different Sodium Diets on Angiotensin II, Serotonin, and Aldosterone Levels in Normal Human Volunteers

Normal female volunteers (age 25-40 years) were studied during the follicular phase of their menstrual cycle, first after a low sodium diet, and then after a high sodium diet, with an intervening period of at least 10 days on an ad libitum sodium intake. Each subject was maintained on either a low sodium (10 mmol/day) or high sodium (200 mmol/day) diet for 4 full days preceding the day of

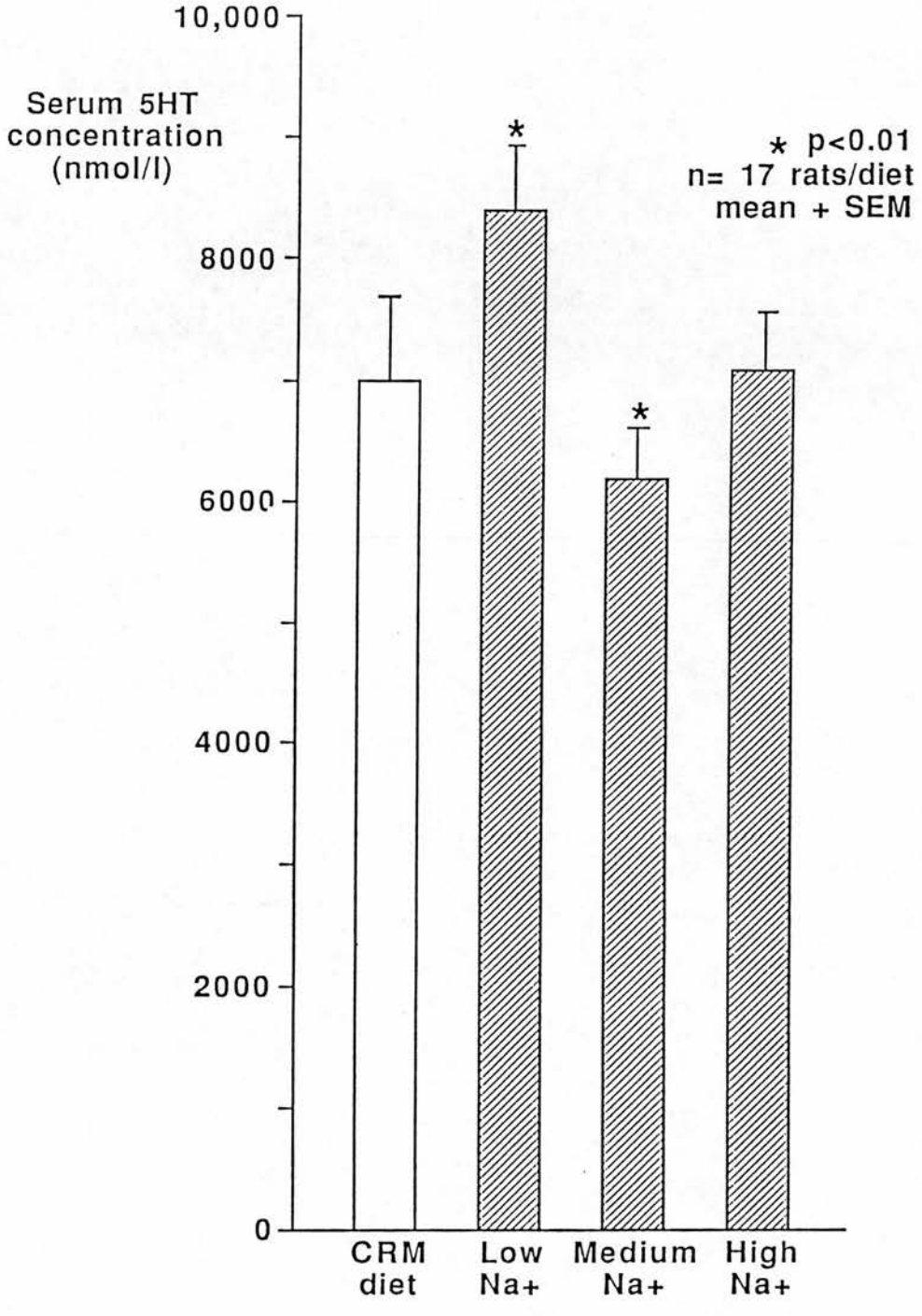


Figure 5.2 The effect of dietary sodium intake on rat serum serotonin levels.

Diet	CRM	Low Na^+	Normal Na^+	High Na^+
Mean (± SD) rat serum aldosterone (nmol/l)	2.3 ±0.7	12.1 ±3.0	1.8 ±0.6	1.7 ±0.6

Table 5.2 Effect of varying sodium intake on rat serum aldosterone levels.

study. Potassium intake was constant at 80 mmol/day. A complete urine collection was made during the 24 h preceding study to verify that the subjects were in sodium balance. Blood was collected on the morning of study, and PRP prepared (as described in section 3.2.5) for aggregation studies and assay of serotonin by RIA; the PRP for serotonin measurement was stored frozen (-20°C) until assayed, which was within one month of the study day. Blood samples (9 ml) were also taken into a solution (1 ml) of EDTA/o-phenanthroline (12.5 and 2.5 mmol/l final concentrations respectively) for measurement of AII (Düsterdieck and M^{c}Elwee, 1971; Morton and Webb, 1985) and into lithium heparin tubes for analysis of aldosterone; plasma was prepared by centrifugation of these samples for 20 min at 1,720 x g at 4°C. Angiotensin II and aldosterone levels were measured (RIA) by the staff of the routine RIA laboratory, Department of Medicine, Western General Hospital, Edinburgh: AII was extracted from plasma using C_{18} "Sep-pak" cartridges (Morton and Webb, 1985), and measured using antisera raised in rabbits against an AII-bovine thyroglobulin conjugate, using [^{125}I]-AII as the tracer. The aldosterone assay was a direct RIA (Al-Dujaili and Edwards, 1981), using sheep antisera raised against aldosterone-3-carboxymethyloxime, and the tracer was [^{125}I]-histamine conjugated to the same oxime. Urinary electrolytes were measured (flame photometry) by the staff of the Metabolic Unit, Western General Hospital. Blood pressures for each subject (supine for 30 min prior to the first reading) were the means of 5 consecutive readings taken using a "Copal" electronic sphygmomanometer. Unless otherwise stated, statistical analyses were

performed using Student's paired t test.

All subjects were in sodium balance on the day of study (see Table 5.3 for summarised results); there was no significant change in urinary potassium excretion, and no change in either the systolic or diastolic blood pressure between the two diets, but plasma levels of both AII and aldosterone fell significantly ($p<0.01$ and $p<0.001$ respectively) when the intake of sodium was increased. There was no significant correlation between the concentration of serotonin and aldosterone (Figure 5.3), or plasma AII and serotonin (Figure 5.4). Significant positive correlations were observed between plasma AII and aldosterone levels (Figure 5.5), and nmol serotonin/l PRP and pmol serotonin/10^8 platelets (Figure 5.6).

There was a significantly ($p<0.01$) greater number of platelets/ml recovered in PRP from volunteers on a low sodium diet compared with a high sodium intake (Figure 5.7); the mean platelet volumes (MPV) and platelet distribution width (PDW) did not change (Figure 5.8). Serotonin concentration (nmol/l PRP) fell significantly ($p<0.01$) when subjects changed from a low to a high sodium intake (Figure 5.9); when expressed as pmol/10^8 platelets, the mean serotonin concentration still fell, but this drop was not significant (Figure 5.10).

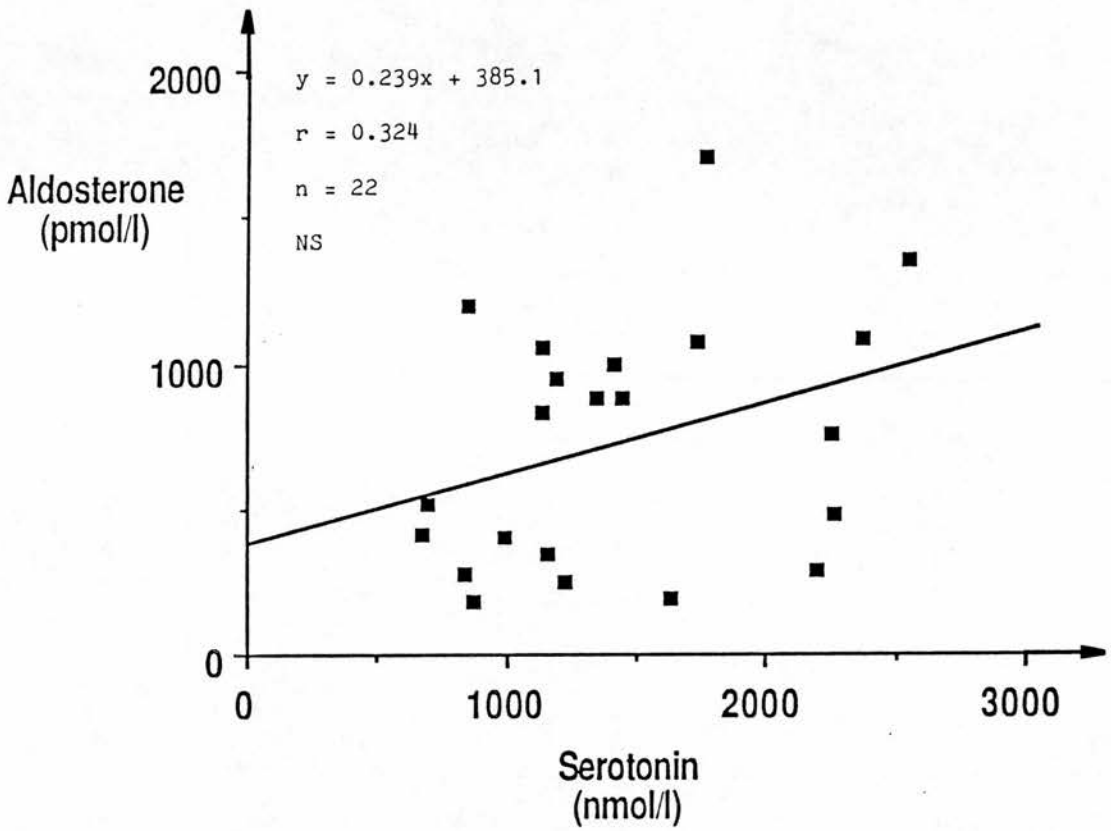
5.4 Effect of Different Sodium Diets on ADP-induced Aggregation of Platelets from Normal Human Volunteers

Aggregation studies were completed as described (section 4.3) within 90 min of venesection using ADP as the aggregating agent at final concentrations of 1 or 4 μmol/l. The rate of aggregation was

Parameter	Low Na^+ Diet	High Na^+ Diet	n	p value
Systolic Pressure (mm Hg)	102.7±5.9	102.7±8.7	12	NS
Diastolic Pressure (mm Hg)	65.2±8.0	62.3±5.0	12	NS
Plasma AII (pg/ml)	35.3±21.6	18.9±17.2	10	p<0.01
Plasma Aldosterone (pmol/l)	1010.0±343.4	362.9±186.6	12	p<0.001
Urinary Na^+ (mmol/24 h)	<20	193.5±25.6	12	--
Urinary K^+ (mmol/24 h)	66.9±21.5	69.1±17.5	12	NS
Platelets (x 10^9/lPRP)	449.0±19.5	370.0±17.5	11	p<0.01
Serotonin (nmol/l)	1642.0±548.4	1241.3±555.9	11	p<0.01
Serotonin (pmol/10^8 platelets)	360.6±93.8	335.1±133.3	11	NS
Aggn. ADP 1 µmol/l	18.6±7.9	30.3±12.6	10	p<0.05
ADP 4 µmol/l	49.9±7.2	68.6±9.3	10	p<0.001
Rate ADP 1 µmol/l	53.3±18.6	62.8±18.1	10	NS
ADP 4 µmol/l	113.0±28.2	116.7±11.5	10	NS

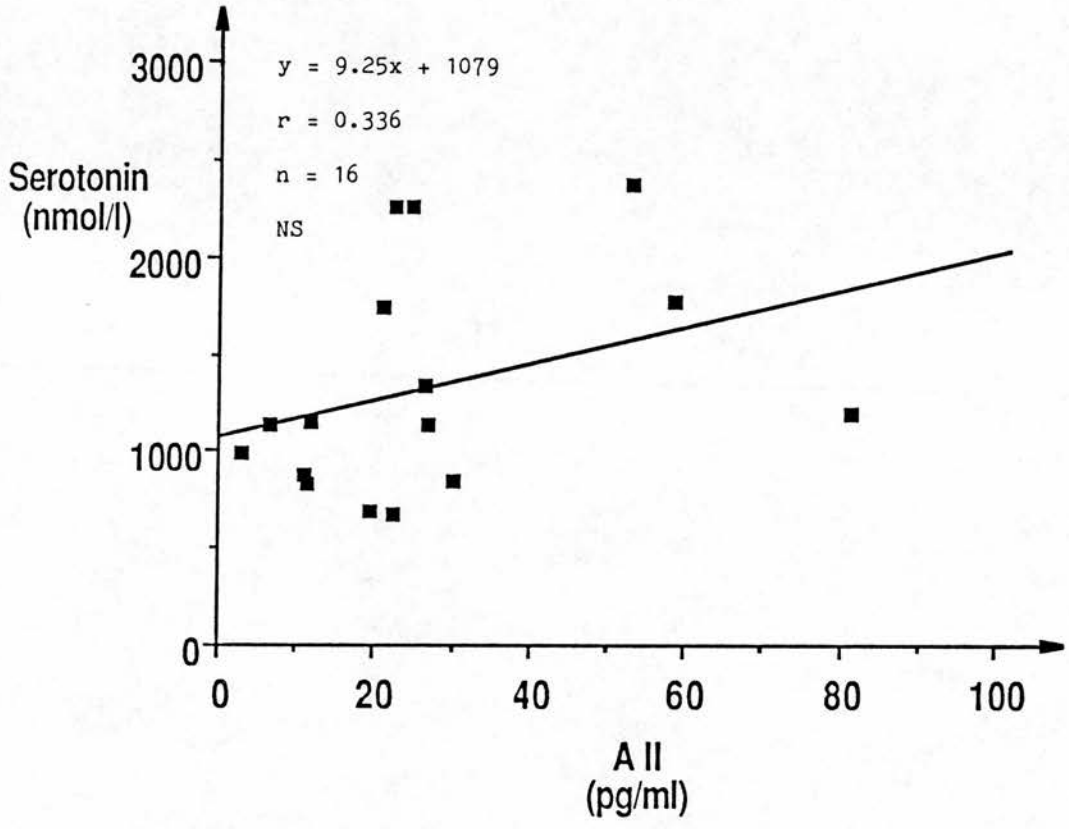
Table 5.3 Summary of results (mean ± SD) on the effect of varying sodium intake in normal female volunteers.

Aggn. = % extent of aggregation
Rate = initial rate of aggregation (% per min)



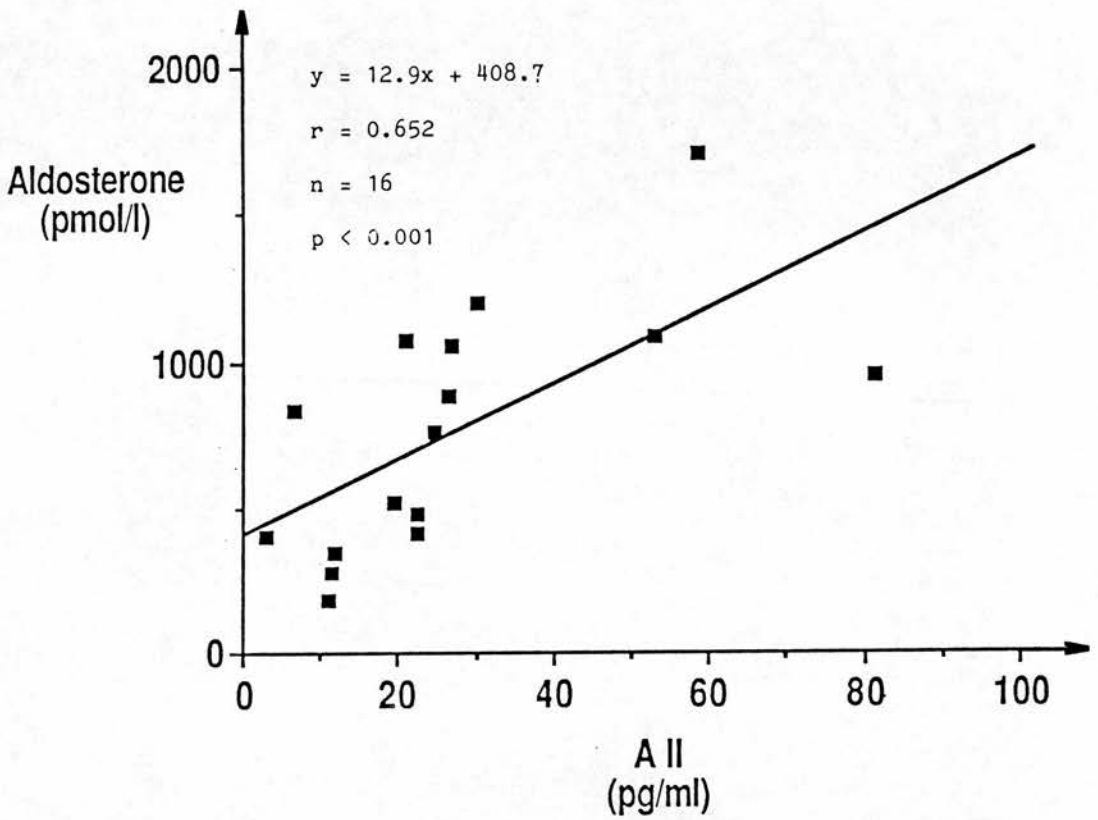
(Analysis by Spearman's rank correlation coefficient: NS)

Figure 5.3 **Correlation between plasma serotonin and aldosterone concentrations in normal females (pooled results from low and high sodium diets).**



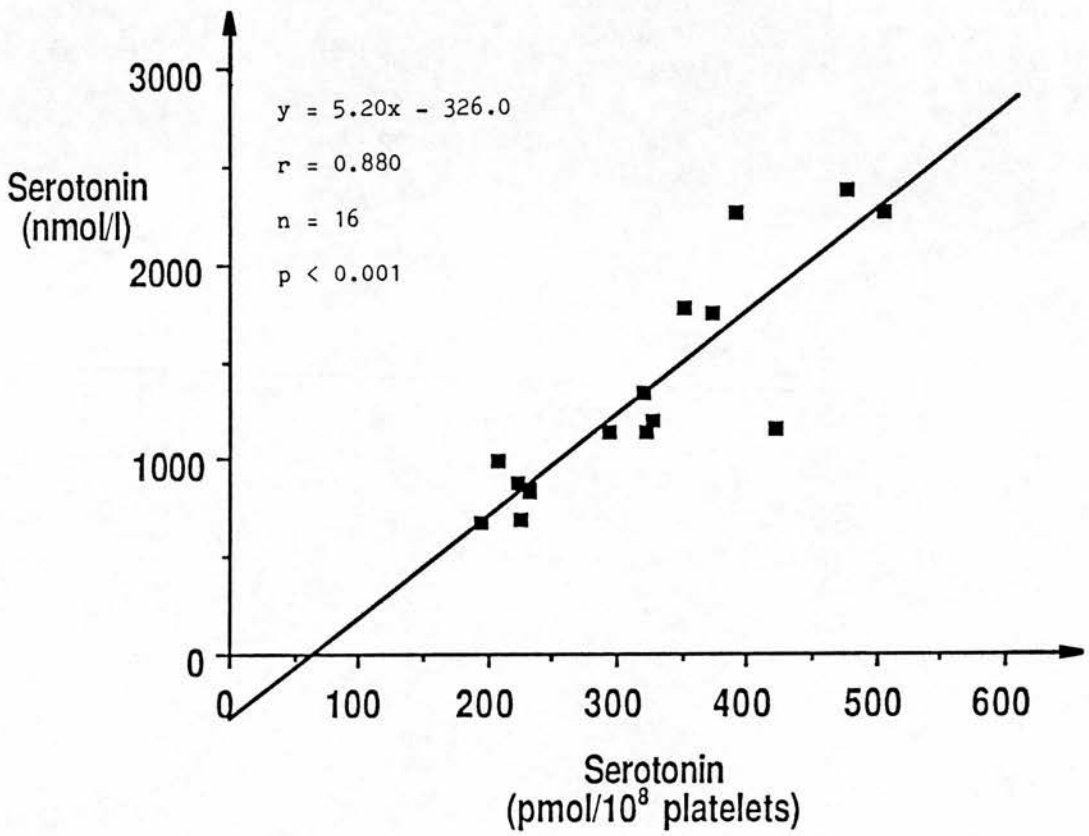
(Analysis by Spearman's rank correlation coefficient: NS)

Figure 5.4 **Correlation between plasma AII and serotonin concentrations in normal females (pooled results from low and high sodium diets).**



(Analysis by Spearman's rank correlation coefficient: $p<0.01$)

Figure 5.5 **Correlation between plasma AII and aldosterone concentrations in normal females (pooled results from low and high sodium diets).**



(Analysis by Spearman's rank correlation coefficient: $p<0.001$)

Figure 5.6 Correlation between pmol serotonin/10^8 platetlets and nmol serotonin/l PRP.

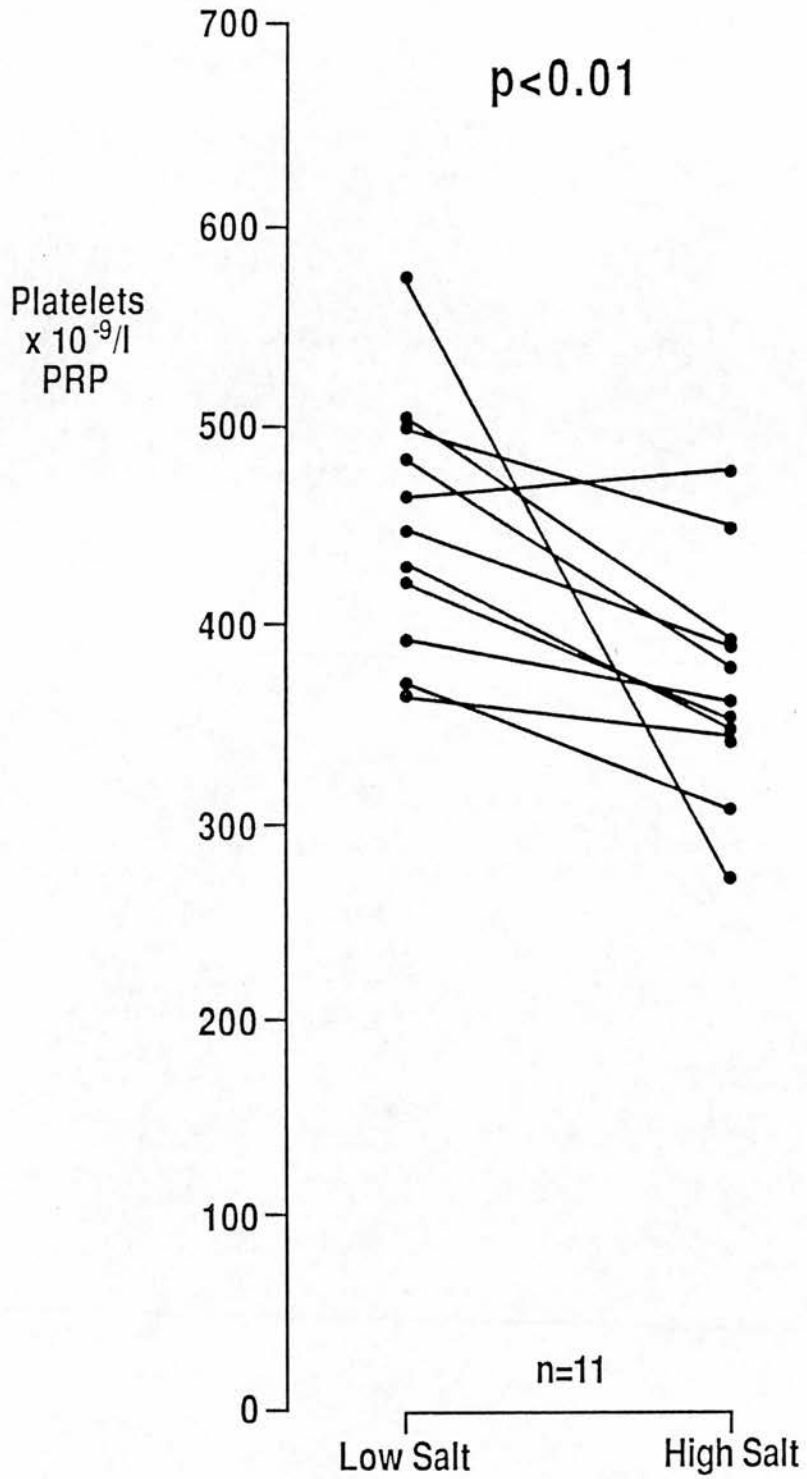


Figure 5.7 Comparison of normal female platelet counts (PRP) on the low or high sodium diet.

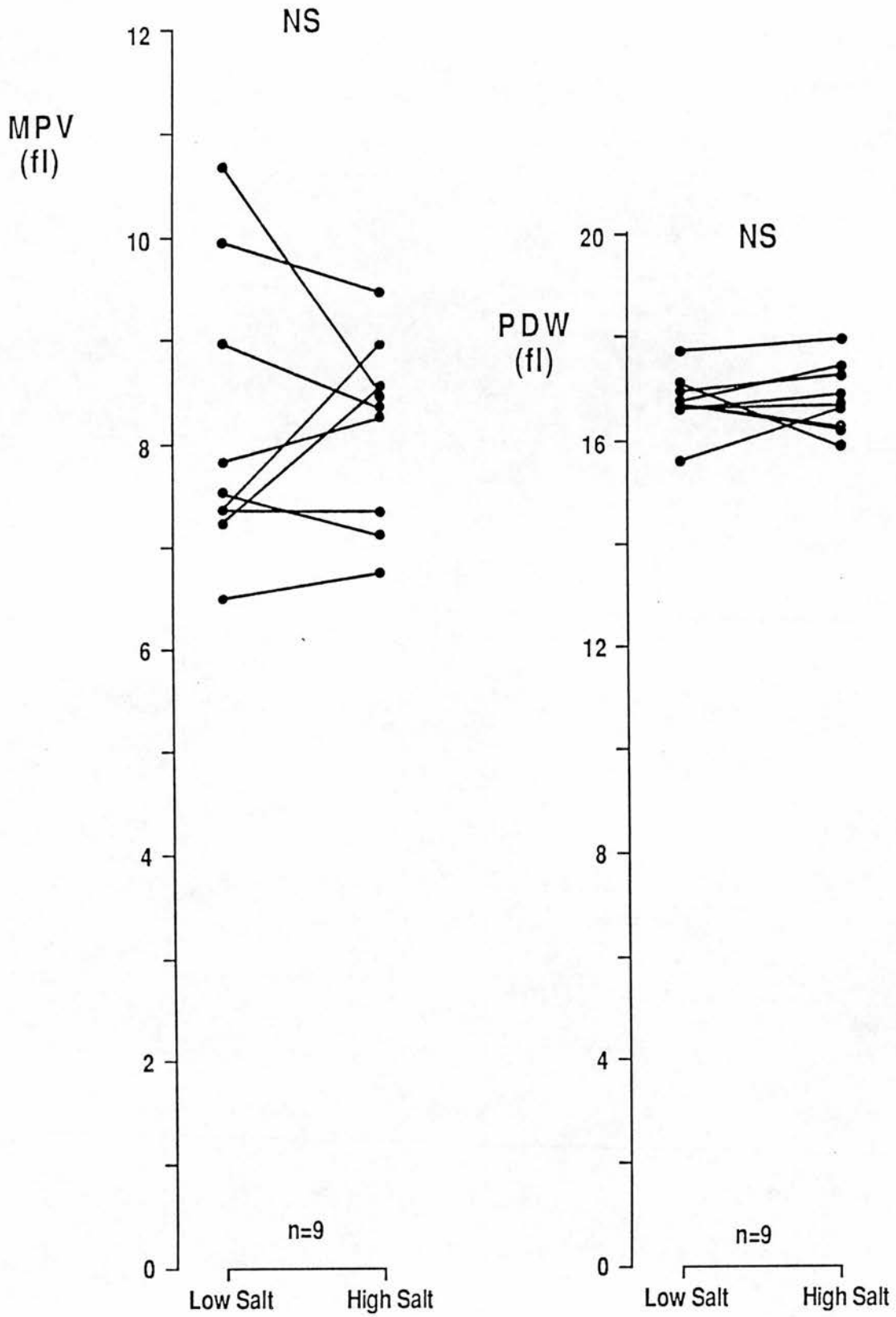


Figure 5.8 Comparison of mean platelet volume (MPV), and platelet distribution width (PDW) in normal female PRP (low or high sodium diet).

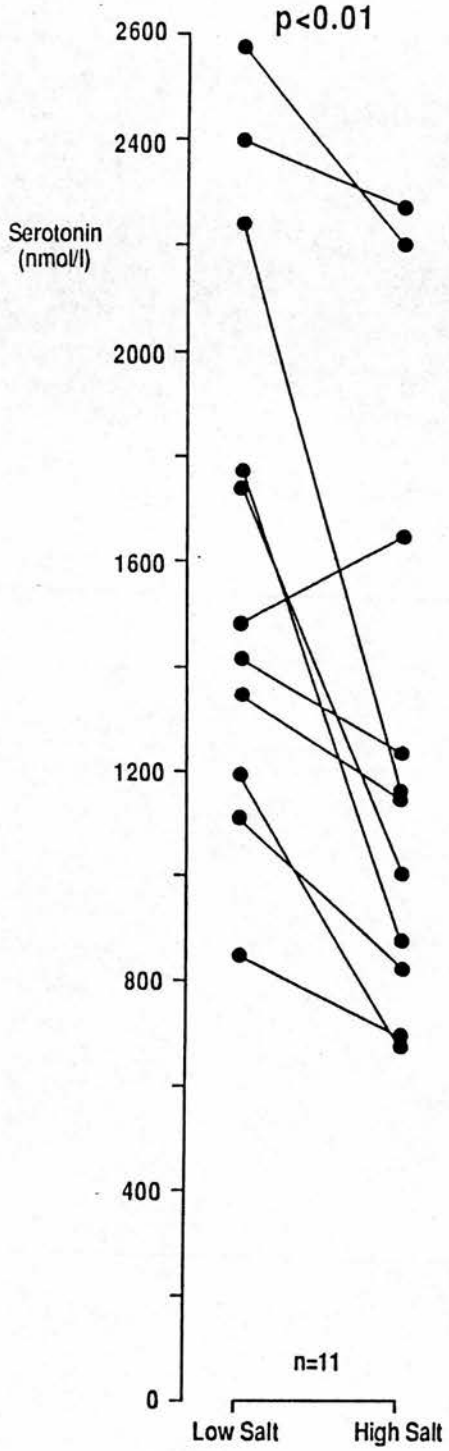


Figure 5.9 **Comparison of PRP serotonin levels (nmol/l) in normal female volunteers on a low or high sodium diet.**

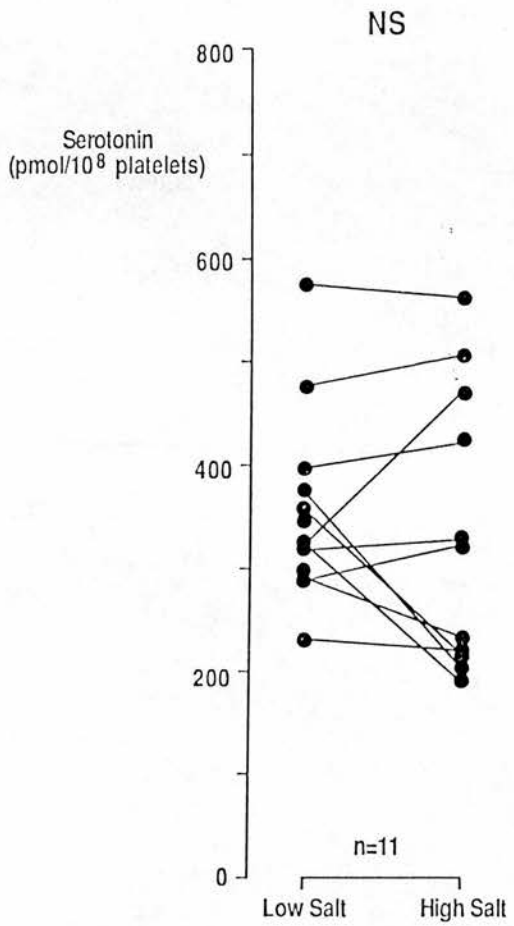


Figure 5.10 Comparison of PRP serotonin levels (pmol/10^8 platelets) in normal female volunteers on a low or high sodium diet.

calculated by measuring the slope of the initial phase of aggregation, and expressing this as % transmission/min, with PPP = 100%, and PRP = 0%. Typical aggregation profiles using platelets from a volunteer initially on a low sodium diet, followed by a high sodium diet, are shown in Figure 5.11. Changing from a low to a high salt intake resulted in irreversible aggregation with 4 μmol/l ADP (Table 5.4), and a significant increase in the extent of aggregation *in vitro* using both 1 and 4 μmol/l ADP (Figure 5.12 and Table 5.3); there was no difference in the initial rate of aggregation between the two salt diets (Figure 5.13 and Table 5.3). A significant inverse correlation was seen between log serotonin concentration (nmol/l PRP) and the extent of aggregation induced by 4 μmol/l ADP (Figure 5.14a). A similar but not significant trend was seen between log serotonin concentration (pmol/10^8 platelets) and the extent of aggregation induced by 4 μmol/l ADP (Figure 5.14b). There was no significant correlation between the measured levels of plasma AII and the extent of *in vitro* aggregation induced by 1 μmol/l ADP (Figure 5.15), but there was a significant ($p<0.02$) correlation between the extent of aggregation induced by 4 μmol/l ADP, and plasma AII concentration (Figure 5.16).

5.5 Effect of adding Ketanserin or Saralasin *in vitro* on the ADP-induced Aggregation of Platelets from Normal Human Volunteers during Different Sodium Intakes

The effect of pre-incubating PRP for 1 min with either the serotonin antagonist ketanserin (1 μmol/l final concentration), or

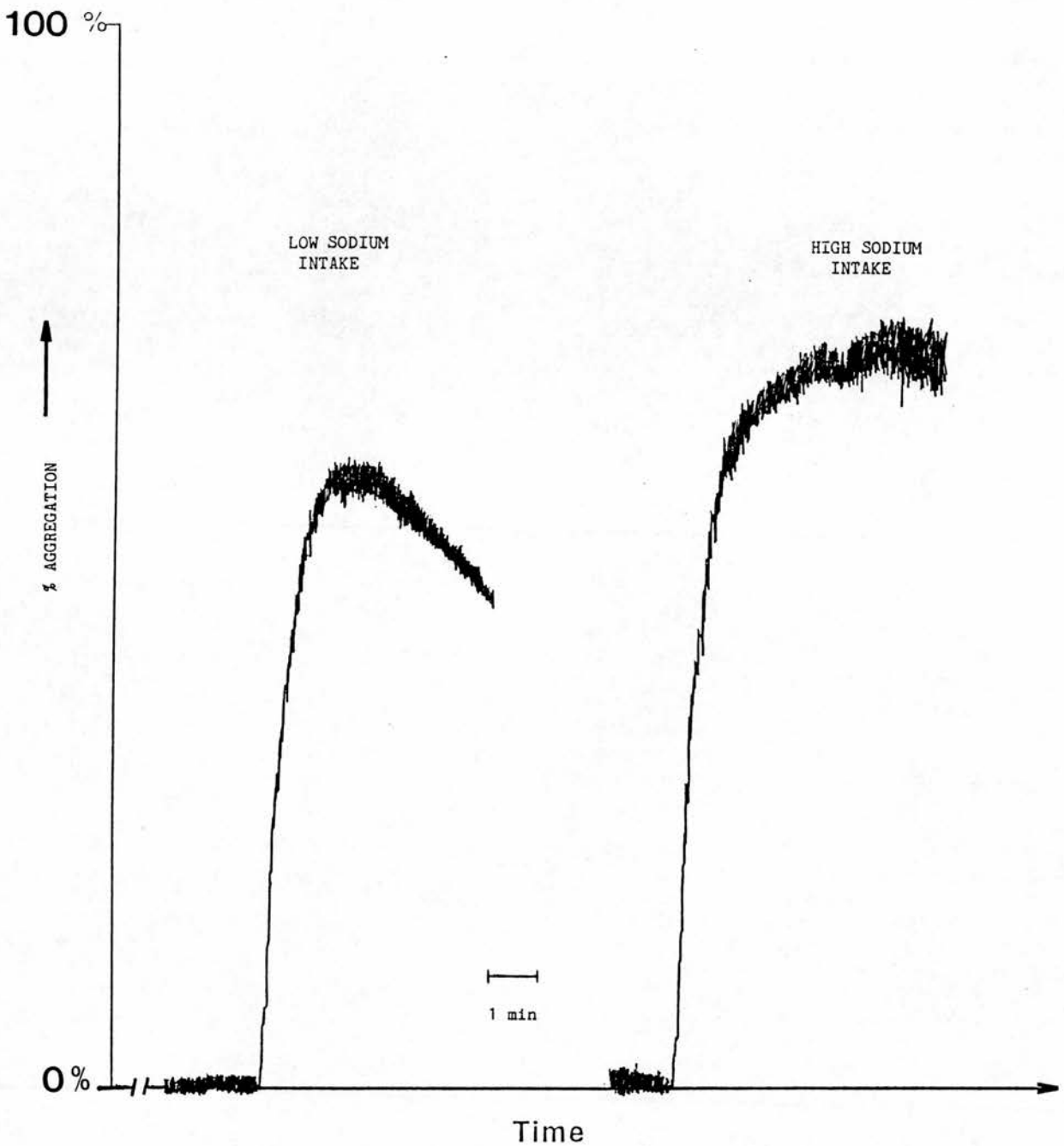


Figure 5.11 Typical platelet aggregation profile obtained using 4 mol/l ADP with PRP from a normal female volunteer on either a low or high sodium diet.

Volunteer	Low Na^+		High Na^+	
	1 μmol/l ADP	4 μmol/l ADP	1 μmol/l ADP	4 μmol/l ADP
1	N	N	N	Y
2	N	N	N	Y
3	N	N	N	Y
4	N	N	N	Y
6	N	N	N	N
7	N	N	N	Y
8	N	N	N	N
9	N	N	N	N
10	N	N	Y	Y
11	N	N	N	N

Table 5.4 **Presence of irreversible aggregation in platelets from normal female volunteers on varying sodium intakes:**

N = no irreversible aggregation

Y = irreversible aggregation present.

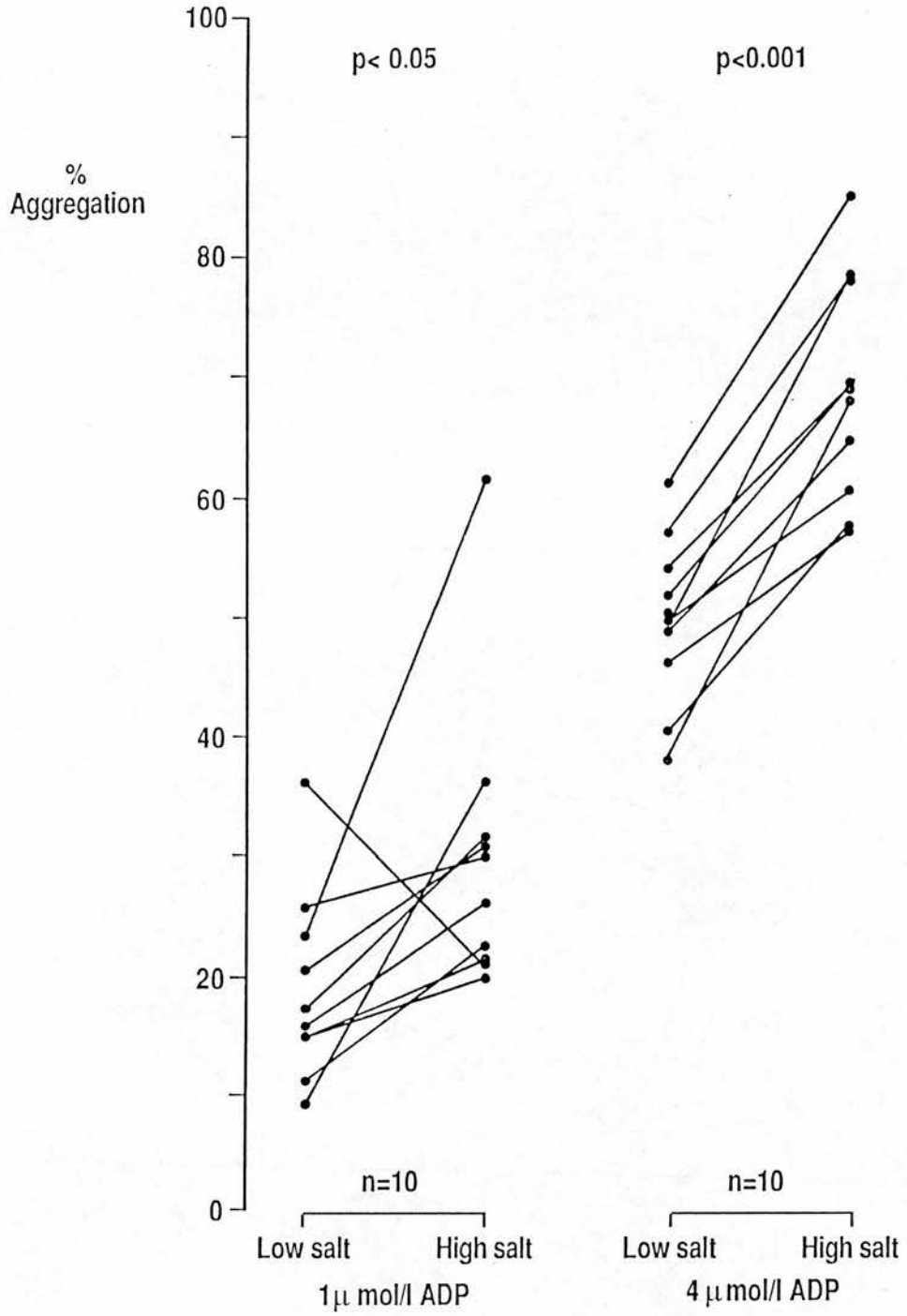


Figure 5.12 **Effect of varying sodium intake on the extent of ADP-induced aggregation in PRP from normal female volunteers.**

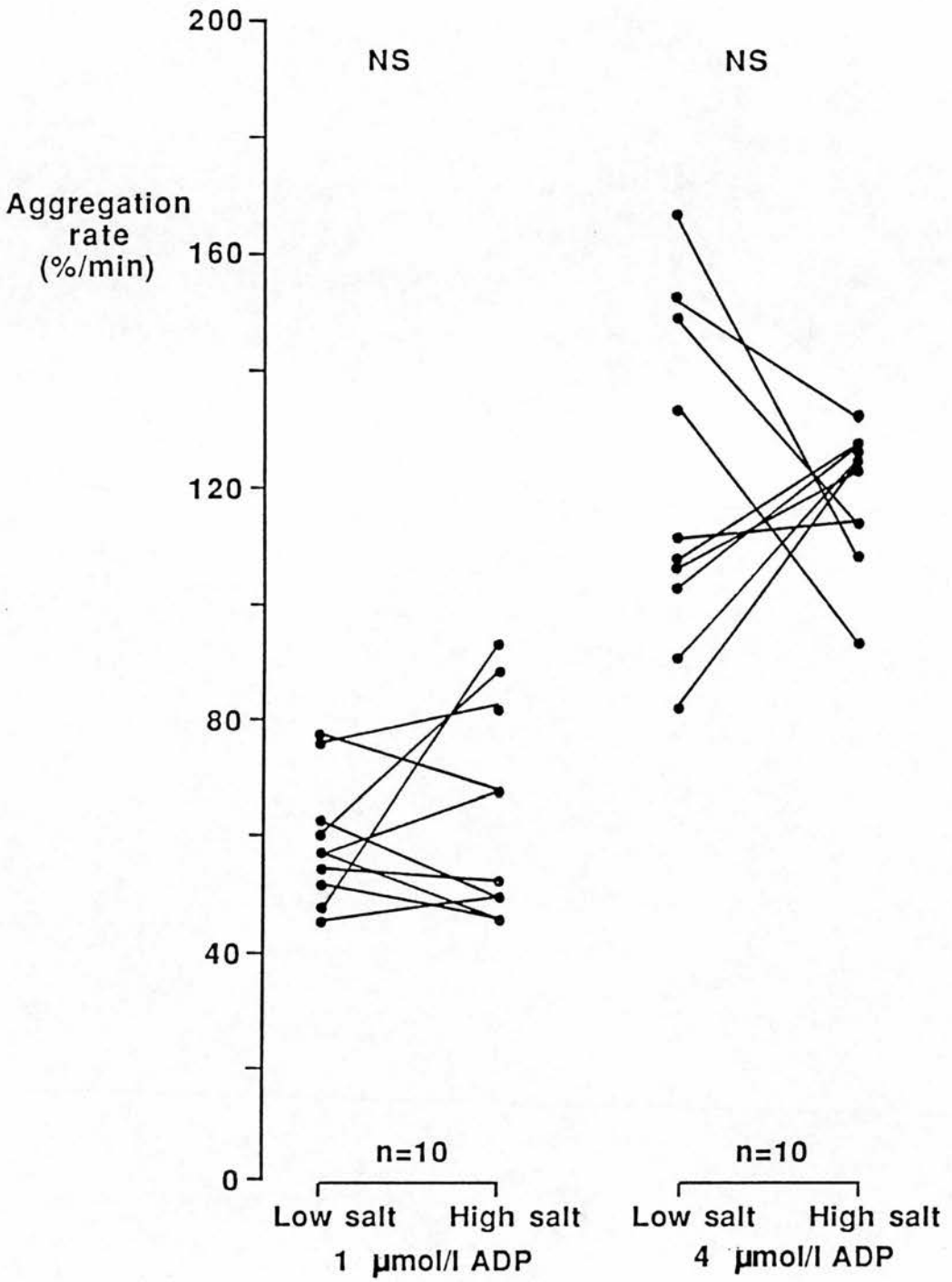
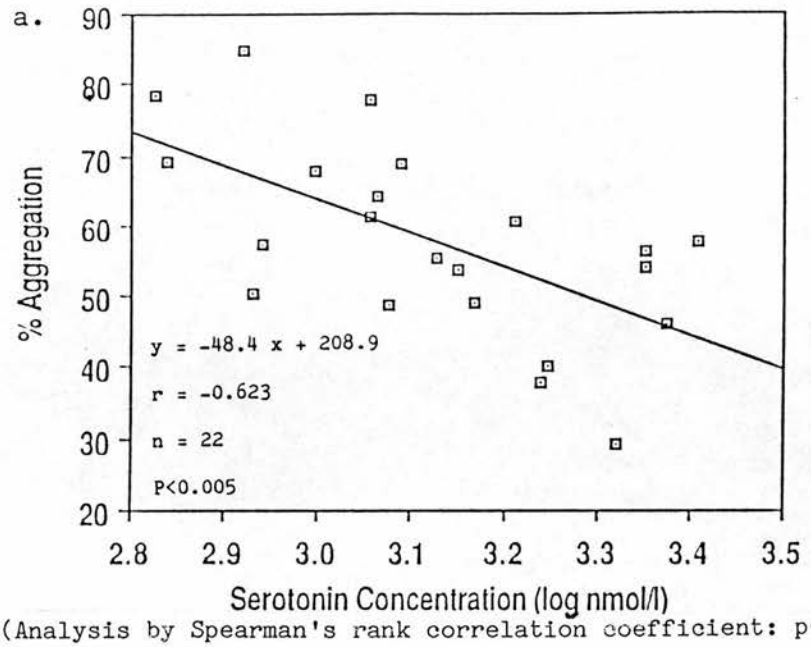
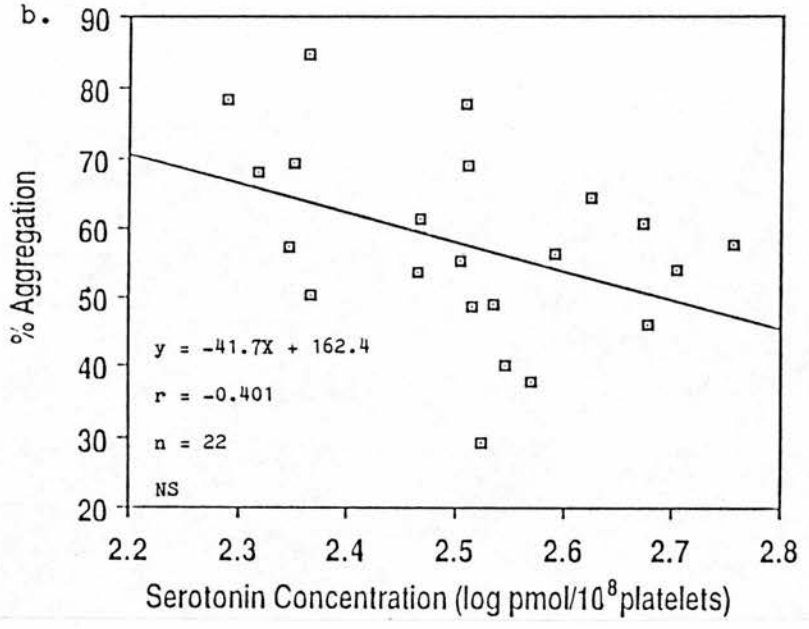


Figure 5.13 Effect of varying sodium intake on the rate of ADP-induced aggregation in PRP from normal female volunteers.



(Analysis by Spearman's rank correlation coefficient: p<0.05)

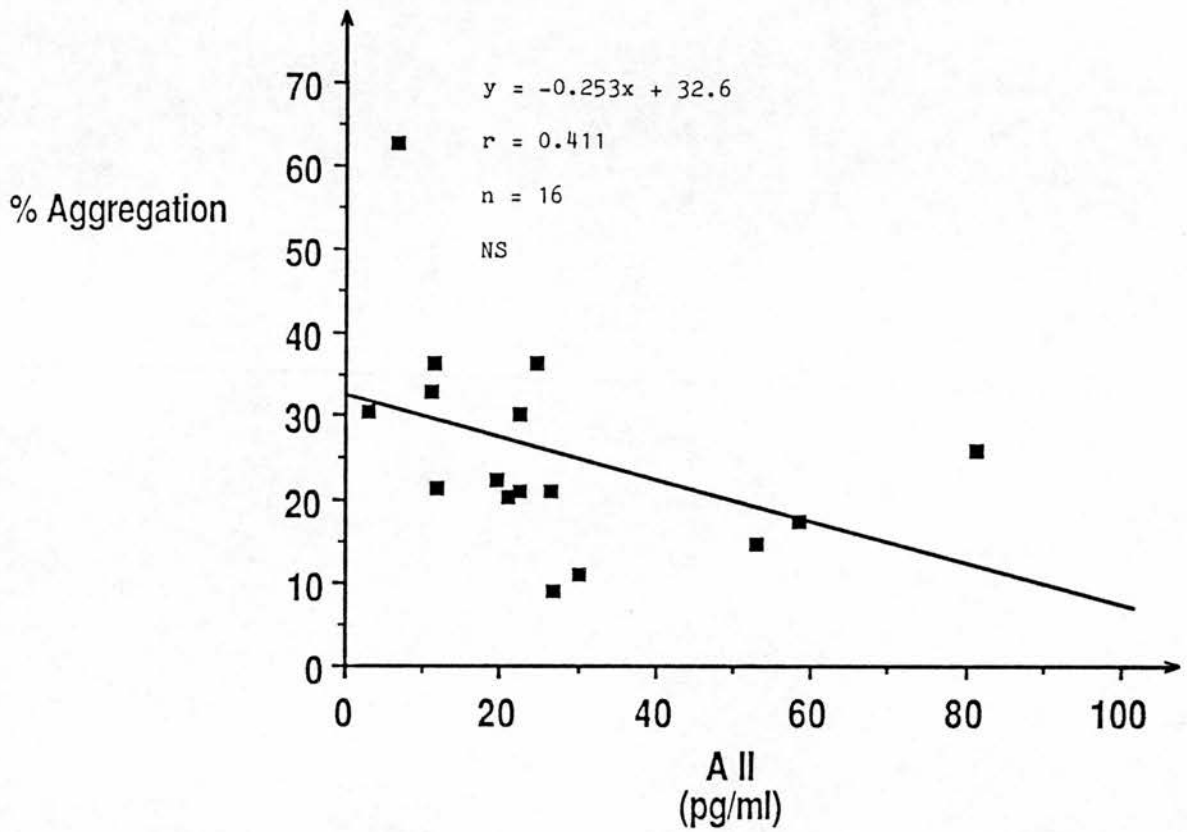


(Analysis by Spearman's rank correlation coefficient: NS)

Figure 5.14 **Correlation between serotonin concentration, and extent of aggregation induced by 4 µmol/l ADP in PRP from normal female volunteers (pooled results from low and high sodium diets):**

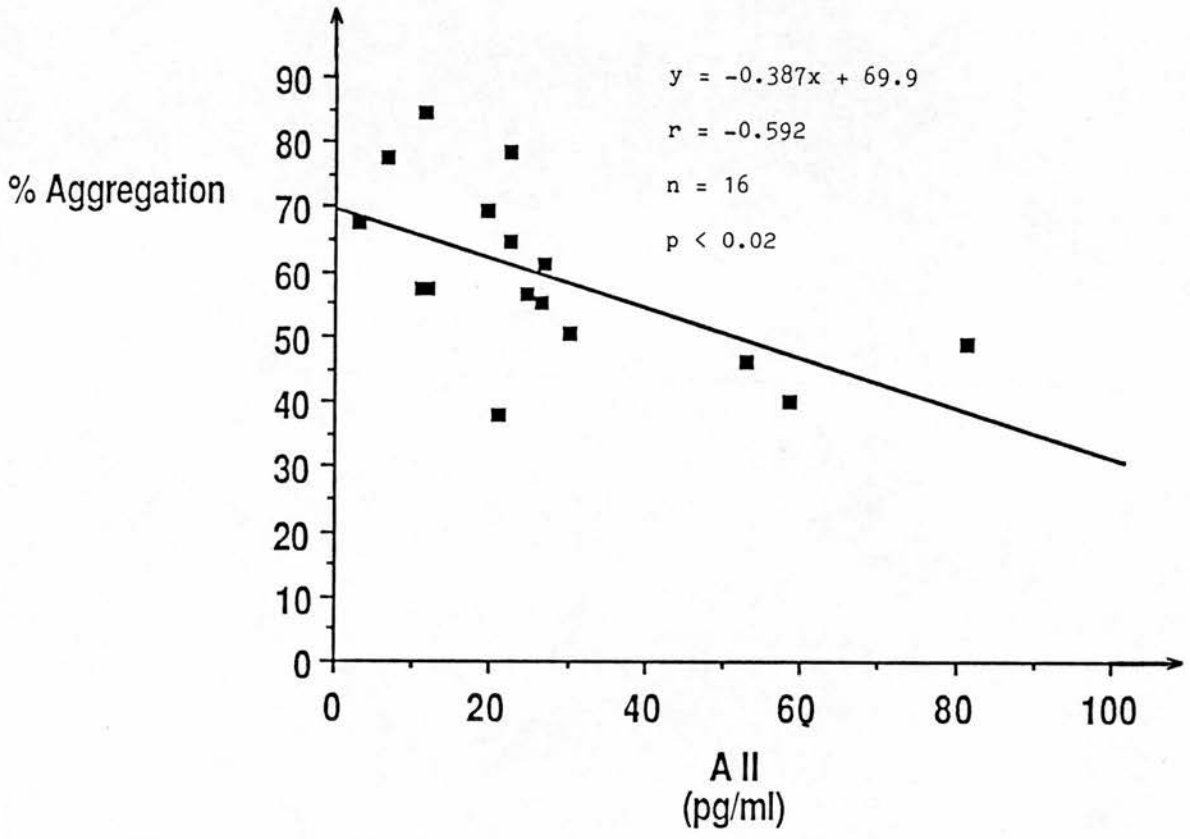
a) % aggregation vs. log nmol serotonin/l

b) % aggregation vs. log pmol serotonin/10^8 platelets



(Analysis by Spearman's rank correlation coefficient: $p<0.02$)

Figure 5.15 Correlation between AII concentration, and extent of aggregation induced by 1 µmol/l ADP in PRP from normal female volunteers (pooled results from low and high sodium diets).



(Analysis by Spearman's rank correlation coefficient: $p<0.02$)

Figure 5.16 Correlation between AII concentration, and extent of aggregation induced by 4 µmol/l ADP in PRP from normal female volunteers (pooled results from low and high sodium diets).

the AII antagonist saralasin (1 nmol/l) was studied as described in section 4.3.

Ketanserin significantly decreased both the extent and initial rate of aggregation induced by 4 μmol/l ADP in PRP prepared from subjects after sodium loading : when the subjects were on a high sodium intake, the extent of aggregation (%) fell from 64.2 ± 7.9 to 55.3 ± 4.4 (mean ± SD; $p<0.02$, control and with ketanserin respectively, n=6) compared with 49.0 ± 8.0 to 47.1 ± 8.7 (mean ± SD; NS, control and with ketanserin respectively, n=7) using PRP taken from the same subjects on a low sodium diet (see also Figure 5.17). The rate of aggregation (%/min) fell from 121.2 ± 7.4 to 107.2 ± 10.3 in PRP from subjects on a high sodium intake (mean ± SD; $p<0.05$, control and with ketanserin respectively, n=6), whereas on a restricted sodium diet, the aggregation rates were 113.1 ± 25.5 and 103.8 ± 25.5 (mean ± SD; NS, control and with ketanserin respectively, n=7; see Figure 5.18). Saralasin had no significant effect either on the extent or the initial rate of aggregation (Figures 5.17 and 5.18), but it was noticed that addition of saralasin *in vitro* caused a rapid decrease in light transmission of the sample (e.g. Figure 5.19), which is usually attributed to the platelets changing shape (Born, 1970). Ketanserin resulted in an initial increase in light transmission, which is presumably due to a dilution effect, since this was also observed when 199/ACD alone was added (Figure 5.19).

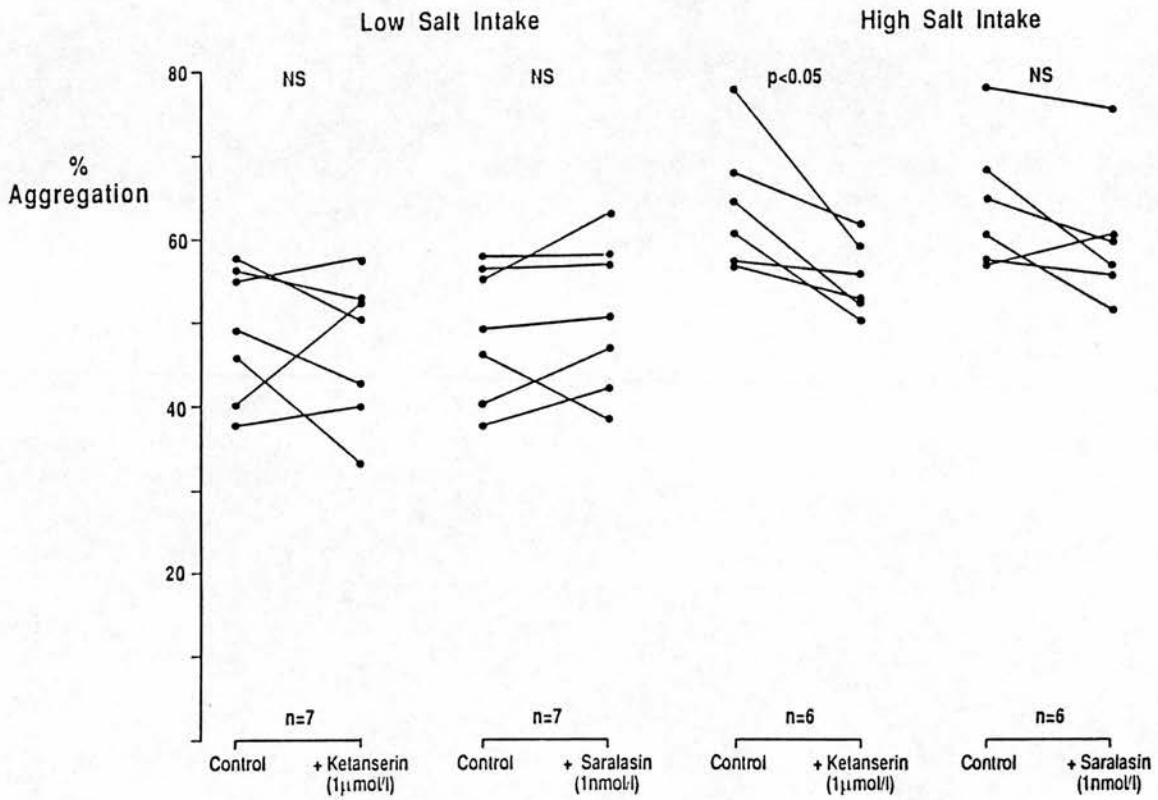


Figure 5.17 Effect of ketanserin (1 μmol/l) and saralasin (1 nmol/l) on the extent of aggregation induced by 4 μmol/l ADP in PRP from normal female volunteers on low or high sodium diets.

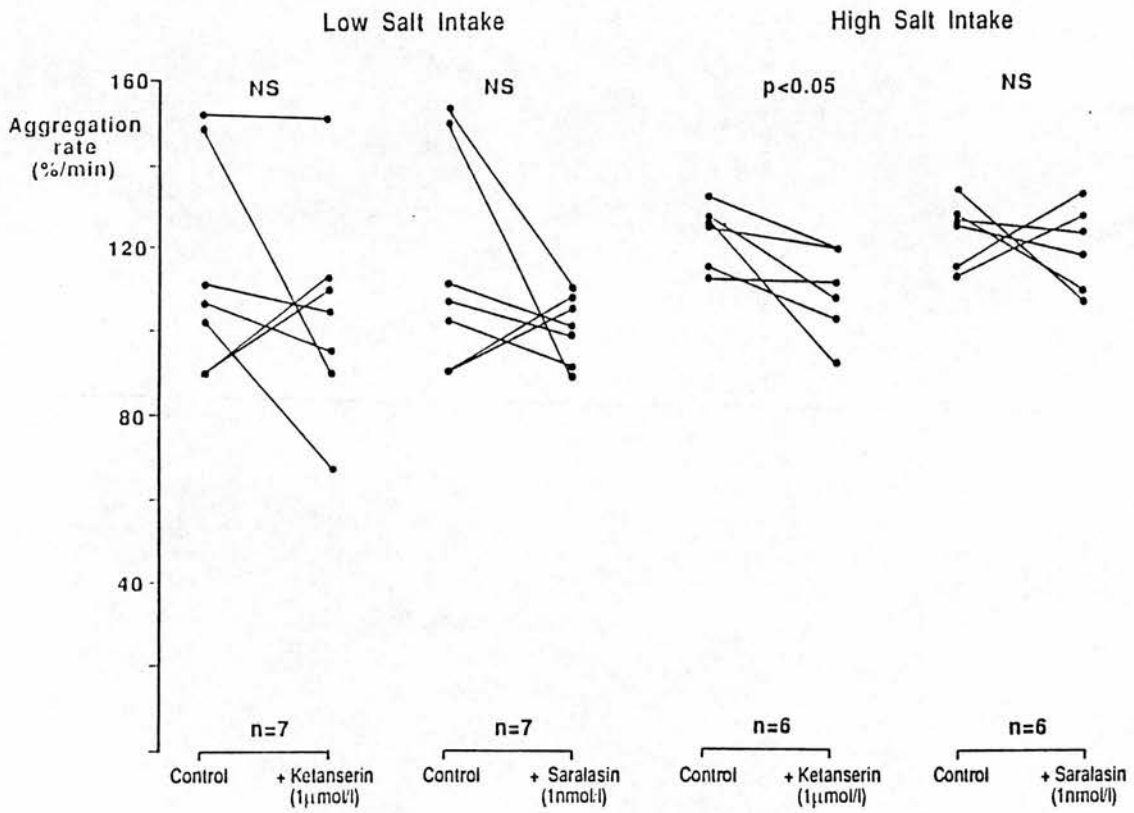


Figure 5.18 Effect of ketanserin (1 µmol/l) and saralasin (1 nmol/l) on the rate of aggregation induced by 4 µmol/l ADP in PRP from normal female volunteers on low or high sodium diets.

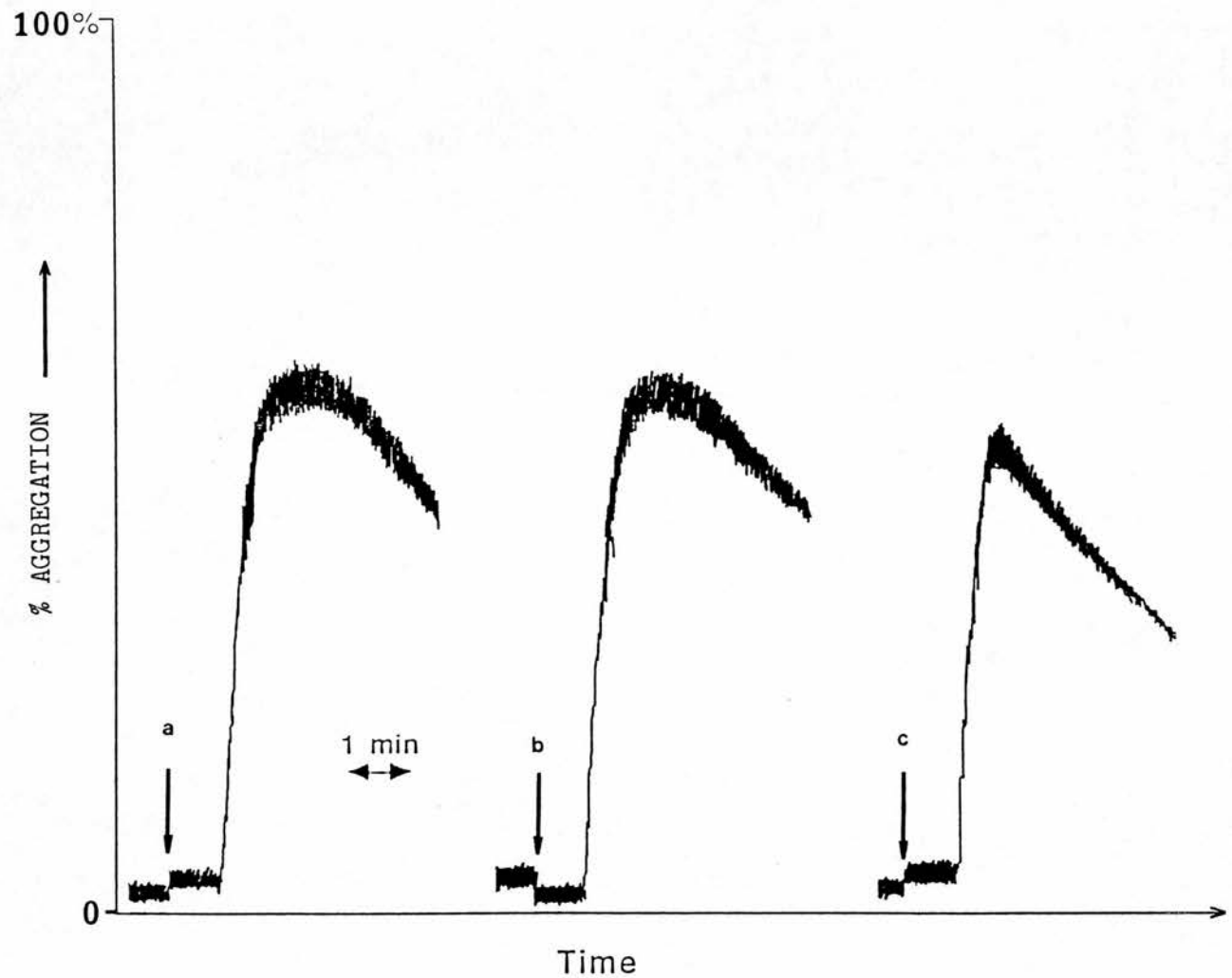


Figure 5.19 Effect of a) Medium 199/ACD, b) saralasin (1 nmol/l), or c) ketanserin (1 μmol/l) on the light transmission of PRP prepared from normal female volunteers on low or high sodium diets.

5.6 Discussion

In the rat model, there was a significant difference in serum serotonin levels between the low and the medium salt diets; given this difference, it was somewhat surprising that further suppression of the renin-angiotensin system by increasing the sodium load resulted in a slight but insignificant rise in serum serotonin; the reason for this apparent anomaly remains unresolved. The aldosterone results show the expected trend, i.e. gradual decrease of serum aldosterone with increased sodium load.

Platelets from volunteers on low-sodium diets (i.e. high plasma AII levels, low platelet AII receptor density) are known to exhibit decreased aggregation in vitro when compared with a high sodium intake (i.e. low plasma AII levels, high platelet AII receptor density): groups studied included normal males, normal males with a high risk of becoming hypertensive (Nara, et al., 1984), and a sub-group of essential hypertensives (Ashida, et al., 1985). Similarly, in Bartter's Syndrome, a disease where blood AII levels are chronically elevated, induced platelet aggregation in vitro is markedly reduced, and can be inhibited further by sodium deprivation (Stoff, et al., 1980; Van Wersh and Pereira, 1983). The results found in the study described here are in accordance with these previous observations, and provide further evidence that AII may modulate platelet function, although the mechanism of the enhanced platelet aggregation seen during a high salt intake remains to be elucidated.

The slight decrease in platelet levels of serotonin between the

low and high sodium diets may reflect an increase in platelet responsiveness and release during a high sodium intake, as suggested by the *in vitro* data, although the difference did not reach statistical significance, possibly due to the small sample number. Furthermore, it must be borne in mind that subtle differences in platelet biochemistry may not have had the opportunity to express themselves: the volunteers were either sodium restricted or sodium loaded for 4 days prior to the study, therefore only approximately half their total population of platelets could have been affected by the diet, as the lifespan of a human platelet is thought to be approximately 10 days (Aster and Jandel, 1964), and platelets once formed have virtually no capacity to synthesise new proteins.

The possibility that an increase in platelet serotonin release occurs during the high sodium state, which then enhances platelet activation is an attractive hypothesis, since this could explain 1) the slight loss in platelet serotonin, 2) the increase in the extent of aggregation, 3) the reduction of the enhanced aggregation by the serotonin antagonist ketanserin, 4) the inverse relationship between platelet serotonin content and induced aggregation, 5) the inverse correlation between AII levels and the extent of induced aggregation (i.e. lowest AII levels during sodium loading), and 6) the lack of irreversible aggregation seen with platelets from most of the low-sodium volunteers (a similar change in the aggregation profile following sodium loading has already been described elsewhere (Ashida, *et al.*, 1985)). Unfortunately, no other markers of platelet release (e.g. β-thromboglobulin, or platelet factor 4) were

measured in this study; it would also have been useful to have measured *in vivo* platelet activation and aggregation, possibly by the method of Wu and Hoak (1974), as this may have indicated whether the low platelet recoveries seen during sodium loading were due to loss of platelets as aggregates during the centrifugation step.

The effects seen with the serotonin antagonist ketanserin and the AII antagonist saralasin are more difficult to interpret, since ketanserin is also thought to have partial α-adrenoreceptor antagonist activity (van Nueten *et al.*, 1981; Marwood and Stokes, 1984), and platelets are known to have α_2-adrenoreceptors (Motulsky and Insel, 1982; Bond and Salmon, 1985); however, it seems that ketanserin acts *via* the α_1- and not the α_2-adrenoreceptor (Kalkman, *et al.*, 1982; Marwood and Stokes, 1984), thus it is anticipated that the effects observed in this study are not due to blockade of adrenaline, a potent inducer of platelet aggregation. Saralasin is known to have a slight agonist activity at high concentrations, at least during *in vivo* infusions in man (MacGregor and Dawes, 1976), so whether the decrease in light transmission seen in PRP incubated with saralasin is due to blockade of the AII receptor or an AII-agonist effect is not known. However, it does suggest that AII may be a factor in regulation of platelet shape.

If, as has been suggested, AII can stimulate the synthesis of serotonin directly (Nahmod, 1978), the net effect during sodium restriction may be twofold: firstly, an AII-induced increase in serotonin levels may result in direct inhibition of platelet responsiveness due to saturation of the serotonin binding sites on

the platelet membrane (Baumgartner and Born, 1968; Baumgartner and Born, 1969). Secondly, if the platelets were less active *in vivo*, then there would be diminished release of platelet-bound serotonin, resulting in an apparent increase in platelet levels of serotonin. However, there is no evidence in this study to suggest that AII can regulate serotonin production. The significant negative correlation between AII levels and *in vitro* aggregation induced by 4 μmol/l ADP does not demonstrate that the level of AII *in vivo* can affect *in vitro* aggregation directly, although the observed trend is in agreement with the findings of Ding, *et al.*, (1985b), where high *in vitro* levels of AII were seen to inhibit the adrenaline-induced aggregation of human platelets, whereas lower doses of AII potentiated the response. It then becomes difficult to explain the report by Someya, *et al.*, (1984), who demonstrated that chronic therapy with the ACE inhibitor captopril significantly reduced *in vitro* platelet aggregation in patients with essential hypertension (the drug itself had no *in vitro* effect).

One possible explanation is that AII may modulate platelet function through prostaglandin production: it has been demonstrated that AII invoked the release of a compound with prostacyclin-like activity from the canine kidney (Shebuski and Aiken, 1980), which would be expected to decrease platelet activity *in vivo*. These workers reported that the effect of the putative prostacyclin lasted for 45 min after the AII infusion, but since prostacyclin in man does not circulate at levels which would be expected to have a biological effect (Blair *et al.*, 1982; Ritter, *et al.*, 1983), and infusions of

prostacyclin in humans (5-10 ng/kg) are reported to decrease ex vivo platelet aggregation for up 30 min post-infusion (Szczeklik, et al., 1978; FitzGerald, et al., 1979) the observed changes in platelet function must be maintained by other, less transient mechanisms, possibly a prostacyclin-induced elevation of platelet cAMP (Szczeklik, et al., 1978) which is known to inhibit the platelet aggregation response by decreasing the available free cytosolic Ca^{2+} (Vermylen, 1978).

The stimulation of prostaglandin production by AII may be indirect: the enzyme (angiotensin converting enzyme, ACE) which produces AII from angiotensin I (AI) also degrades bradykinin (Erdös, 1975), a peptide which stimulates prostaglandin synthesis (McGiff, et al., 1972); an increase in AII production (e.g. in salt restriction, or Bartter's Syndrome) may mean that the active site on the ACE molecule is occupied by AI in preference to bradykinin. This would lead to an increase in bradykinin, and hence prostaglandin production, and possibly decreased platelet aggregation. Similarly, captopril binds to the ACE molecule, thus blocking the production of AII, but also inhibiting the degradation of bradykinin (Mullane and Moncada, 1980), therefore having the same effect on platelet function as low salt or chronic elevation of AII production.

Chapter 6

Serotonin and Cadiovascular Diseases

6.1 Introduction

There have been many reported differences in biochemistry and behaviour between platelets isolated from normal subjects and from patients with essential hypertension (for a review, see De Clerck, 1986). There is also some evidence to suggest that serotonin may be a factor involved in the pathogenesis of essential hypertension, both in man and in experimental animals: in patients with essential hypertension, treatment with the serotonin $5HT_2$-receptor antagonist ketanserin resulted in a significant decrease in blood pressure (De Cree, et al., 1981; Wenting, et al., 1984), while blood platelets from patients with essential hypertension, or from spontaneously hypertensive rats, have been reported to possess an impaired serotonin uptake mechanism (Bhargava, et al., 1979; Palmero, et al., 1982; Kamal, et al., 1984a and 1984b; Sharma, et al., 1985), decreased platelet levels of serotonin (Bhargava, et al., 1979; Kamal, et al., 1984a and 1984b; Sharma, et al., 1985), and an apparent increase in plasma "free" serotonin (Palmero, et al., 1982; Sharma, et al., 1985). Also, when compared with normal platelets, platelets from patients with essential hypertension have increased free cytosolic calcium (Le Quan Sang and Devynck, 1986), and release [^{3}H]-serotonin at lower doses of thrombin (Valtier, et al., 1986). It has been suggested (Bhargava, et al., 1979) that the observed impaired uptake of a neurotransmitter (i.e. serotonin) into blood platelets may reflect abnormalities in neuronal control of the cardiovascular system, since platelets are thought to be a good model of the adrenergic neurone (Abrams and Soloman, 1969) as both types of

cell have many similar biochemical properties (see Table 6.1).

There are several studies which describe abnormal platelet activity in heart disease: elevated levels of plasma β-thromboglobulin (BTG), an indicator of platelet activation and release, have been reported (Mehta and Mehta, 1982; Pumphrey and Dawes, 1982; Pengo, et al., 1985); increased platelet aggregation and an elevation of thromboxane A_2 (TXA_2) production have also been described (Schwartz, et al., 1980; de Boer et al., 1982). Both TXA_2 and serotonin are released from platelets during aggregation, and both are potent vasoactive compounds, so it is conceivable that these compounds may play a role in the development, if not the initiation, of cardiovascular disease. The relationship between plasma serotonin levels and heart disease is not well documented, which is surprising given the body of evidence for abnormal platelet behaviour in this group of patients, and the potent vasoactive effects of serotonin.

6.2 Comparison of Serotonin Levels and [^{3}H]-Serotonin Uptake in Patients with Essential Hypertension and Normal Controls.

PRP was prepared from males clinically diagnosed as suffering from essential hypertension, or normal male controls. All patients and controls had refrained from taking any medication with known or suspected anti-platelet activity, for the preceding 7 days. Platelet serotonin content was measured by HPLC, and blood pressure by a "Copal" electronic sphygmomanometer (mean of 3 consecutive readings). The uptake of [^{3}H]-serotonin was measured in the 7 hypertensive patients, and 8 of the 12 normal volunteers; PRP platelet count was

Property	Platelets	Synaptosomes
Nucleus	No	Yes
Synthesis of serotonin	No	Yes
Limiting membrane	Yes	Yes
Mitochondra	Yes	Yes
Monoamine oxidase	Yes	Yes
Active transport of serotonin	Yes	Yes
" " " dopamine	Yes	Yes
" " " noradrenaline	Yes	Yes
Storage of biogenic amines in granules	Yes	Yes
Release of stored amine by reserpine	Yes	Yes

Table 6.1 Some similarities between platelets and synaptosomes (after Sneddon, 1973).

determined by Coulter counter in the Haematology Department, Western General Hospital. All results were analysed initially by the Student's *t* test, and later by Wilcoxon's rank sum test.

The results of the study are summarised in Tables 6.2 and 6.3. The systolic and diastolic blood pressures were elevated significantly in the hypertensive group ($p<0.001$ for both, Student's *t* test), and PRP serotonin concentration (nmol/l) in the hypertensive group (n=7) was significantly lower ($p<0.05$; Student's *t* test) than the control group (n=12). No other significant differences were observed between the two groups studied using either the Student's *t* test, or Wilcoxon's rank sum test. When the control group was reduced to n=8, although the trend was the same, no significant difference was found in the serotonin levels between the two groups (Table 6.3) using either of the statistical tests.

6.3 Serotonin Levels in Patients with Cardiac Failure Before and After Captopril Therapy.

A group of patients (4 male, 1 female) with heart failure (New York Heart Association Class III-IV) were selected for study. These patients were diagnosed as suffering from cardiomyopathy on the basis of clinical assessment and echocardiography, and had failed to respond to conventional diuretic therapy (frusemide, 80 - 120 mg/day). Patients were each studied on two separate days, and blood samples were taken on each day for analysis of serotonin levels by RIA. On the first day, blood was collected from the femoral vein *via* a French size 8 catheter (2.64 mm internal diameter) which had been

Parameter	Normals n=12	Essential Hypertensives n=7	p value
Age (years)	39.5 ±13.3	51.3 ±10.5	NS
Systolic Pressure (mm Hg)	128.3 ±11.5	163.4 ±19.5	< 0.001
Diastolic Pressure (mm Hg)	77.6 ±10.8	109.6 ±6.2	< 0.001
Platelets (x 10^9/l)	445.9 ±109.7	376.3 ±152.9	NS
Serotonin (nmol/l PRP)	1,617.6 ±582.2	1,029.7 ±440.9	< 0.05
Serotonin (pmol/10^8 platelets)	372.5 ±147.3	272.9 ±65.9	NS

Table 6.2 Comparison of results (mean value ± SD) from normal males, and males with essential hypertension; p value from Students t test.

Parameter	Normotensive n=8	Hypertensive n=7	p value
Age (years)	43.7 ±14.3	51.3 ±10.5	NS
Systolic Pressure (mm Hg)	130.0 ±12.5	163.4 ±19.5	< 0.001
Diastolic Pressure (mm Hg)	77.6 ±13.1	109.6 ±6.2	< 0.001
Platelets (x 10^9/l)	430.6 ±104.1	376.3 ±152.9	NS
Serotonin (nmol/l PRP)	1,469.1 ±545.9	1,029.7 ±440.9	NS
Serotonin (pmol/10^8 platelets)	352.1 ±155.8	272.9 ±65.9	NS
V_{max}	133.6 ±87.0	114.1 ±54.3	NS
K_m	570.3 ±207.5	521.6 ±306.4	NS

Table 6.3 Comparison of results and parameters of serotonin uptake (mean value ± SD) from normal males, and males with essential hypertension; p value from Students t test.

indwelling for <30 min. Samples were taken prior to, and 1 h after administration of captopril (25 mg orally). The 1 h time point was chosen for study since the maximum blood concentration of captopril occurs 45 - 60 min after oral administration (Shaw, et al., 1985). Thereafter, each patient received 25 mg captopril orally three times daily, and continued with frusemide 40 - 80 mg/day as maintenance therapy. Re-admission for the second study day was after at least one month of therapy. On the second study day, blood was taken from the antecubital vein using a 19 gauge needle as described (3.2.5).

The results are summarised in Table 6.4. There was no significant increase in plasma serotonin concentration within 1 h of receiving captopril (Student's t test; Figures 6.1 and 6.2). Chronic captopril therapy, however, resulted in an increase in total serotonin (i.e. nmol/l PRP; $p<0.05$, Student's t test), but not in platelet serotonin (pmol/10^8 platelets; Figures 6.1 and 6.2). There was no change in PRP platelet count following captopril therapy. Further analyses of these data by the Wilcoxon rank sum test failed to reveal significant differences between any of the measured parameters.

6.4 Discussion

Although there was no significant difference in age between the controls and the patients with essential hypertension the two groups were poorly age-matched, and may indeed be biologically different. It is not clear whether this would affect the serotonin levels, since little work has been done in this area; however, it is known that the

a)

Parameter	Pre-captopril	Post-captopril (1 h)	n	p
Serotonin (nmol/l)	1561.5±316.9	1573.1±359.5	5	NS
Serotonin (pmol/10^8 platelets)	322.4±129.1	420.0±201.4	4	NS
PRP platelet count (10^9/l)	485.0±169.0	393.2±168.0	5	NS

b)

Parameter	Pre-captopril	Post-captopril (>1 month)	n	p
Serotonin (nmol/l)	1561.5±316.9	2352.8±597.8	5	p<0.05
Serotonin (pmol/10^8 platelets)	335.8±115.7	506.2±187.1	5	NS
PRP platelet count (10^9/l)	485.0±169.0	499.4±87.8	5	NS

Table 6.4 **Paired mean (± SD) serotonin levels and PRP platelet counts in heart failure patients before starting captopril therapy, and after**

a) 1 h, or
b) >1 month of captopril therapy.

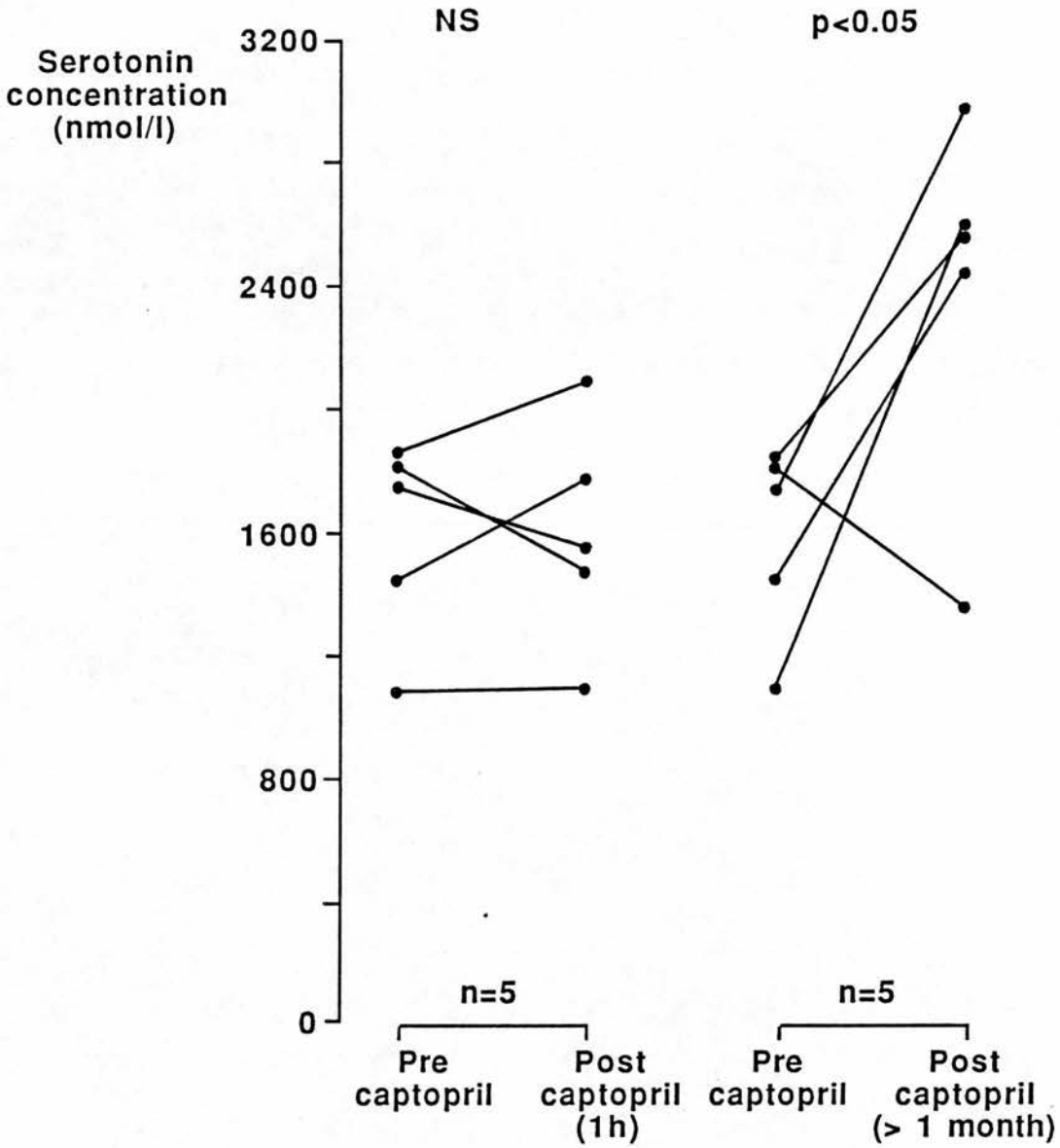


Figure 6.1 Effect of captopril therapy on serotonin levels (nmol/l PRP) in patients with heart failure.

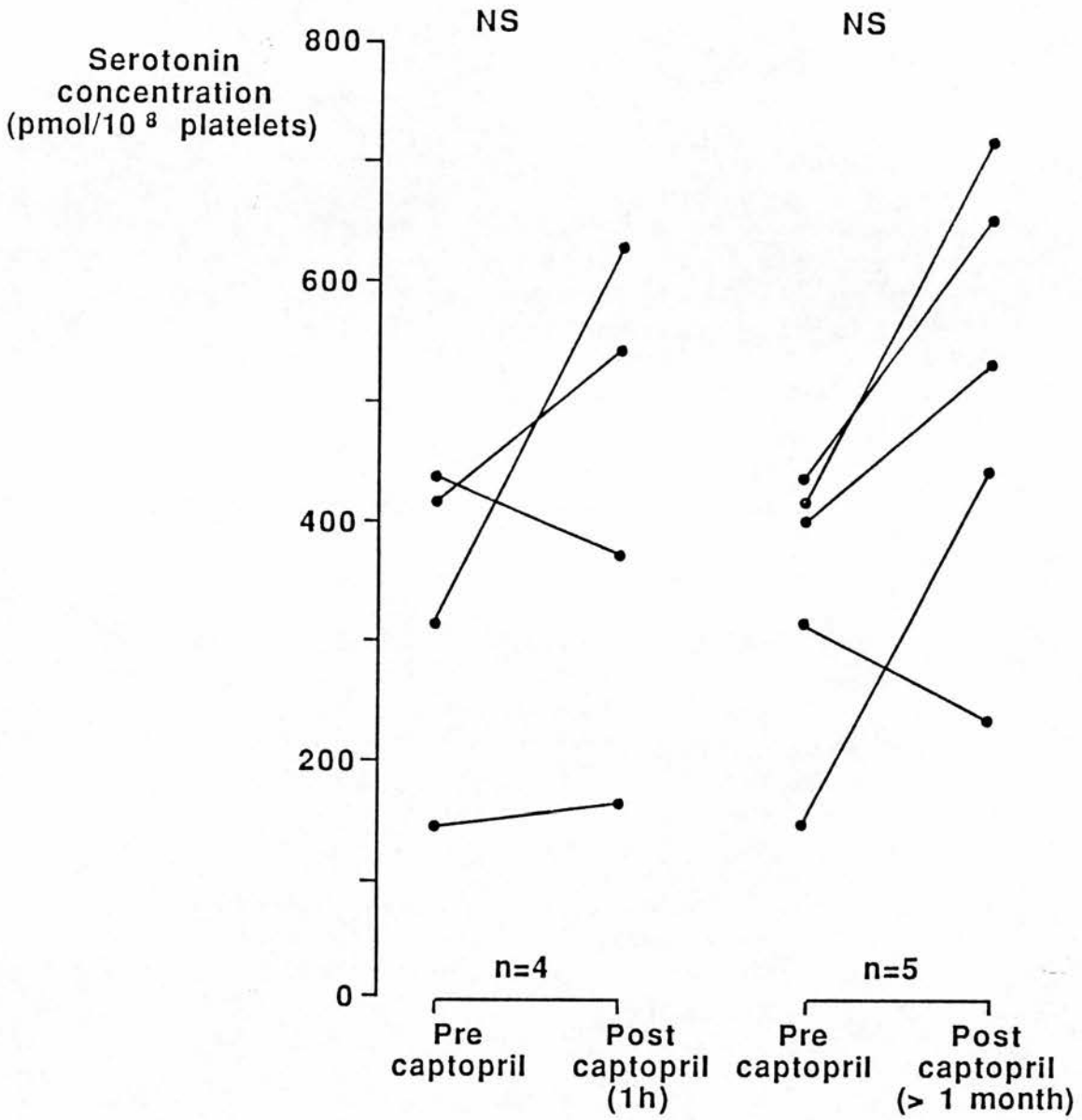


Figure 6.2 Effect of captopril therapy on serotonin levels ($pmol/10^8$ platelets) in patients with heart failure.

plasma levels of β-thromboglobulin (BTG) an indicator of platelet release, do increase with age (Ludlam, 1979). There was no significant correlation between age and serotonin concentration (nmol/l PRP) in the control group (serotonin = 1971.9 - 9.0 x age; NS; n=12), therefore it seems unlikely that any decrease in total serotonin seen in these data would be due to an age-related increase in the release reaction. Both systolic and diastolic blood pressure are known to increase with age (Hamilton, et al., 1954), but the group of patients studied here had already been selected on the basis of having abnormally high blood pressure for their age group.

No significant differences were seen in the kinetics of [^{3}H]-serotonin uptake between platelets from patients with essential hypertension, and the normotensive control group. There may be several reasons why the results obtained in this study differ from those obtained by other workers (e.g. Bhargava, et al., 1979; Kamal, et al., 1984a and 1984b). The sample size is small, so that a small difference between the two populations may not be statistically significant (Kamal, et al., (1984a) saw significant differences with a sample size of 16-17 for the controls or patients). Also, it is possible that the population of patients suffering from essential hypertension is itself subdivided: Ashida, et al., (1985) demonstrated that a group of essential hypertensives who changed from a high to a low sodium diet could be divided into two types, namely those whose blood pressure fell ("responders") and those whose blood pressure remained unchanged ("non-responders"). In the "responders" there was a significant increase in platelet α_2-adrenoreceptors during

salt loading, but there was no such increase in the non-responders. The methodology used to study platelet [^{3}H]-serotonin uptake in this thesis may also have influenced the results: if the alteration in uptake kinetics is due to an endogenous plasma factor rather than a platelet defect, dilution of PRP in KHS may have reduced the concentration of this putative inhibitor to such an extent that it has no observable effect on uptake. Endogenous imipramine-like factors have been described in human plasma (Angel and Paul, 1984; Brusov, et al., 1985), and it is known that other plasma components can reduce the uptake of serotonin into platelets (e.g. derivatives of phenylethylamine (Richter and Smith, 1974), and derivatives of tryptamine (Born, et al., 1972; Baker, et al., 1980)). Furthermore, in migraine, where, during an attack, platelet levels of serotonin are decreased (Hilton and Cumings, 1971) and there is impaired platelet serotonin uptake (Coppen, et al., 1979), Launay and Pradalier (1985) demonstrated that the inhibition of uptake is probably due to an unidentified plasma factor rather than a platelet defect. It is conceivable a similar situation may exist in essential hypertension. It is unlikely that the reported inhibition of uptake of radioactive serotonin into platelets is due entirely to endogenous "free" serotonin in the samples, since this would not result in a change in V_{max}, as the inhibition would be truly competitive.

The difference in PRP serotonin concentration between the controls and the patients with essential hypertension was not significant after correcting for the platelet count; this may be due in part to

inclusion of further errors (i.e. from platelet counting) affecting overall precision, but it also reflects the higher platelet counts seen in normal PRP compared with PRP prepared from patients with essential hypertension. This probably does not reflect differences in whole blood platelet numbers, since essential hypertension patients are reported to have a normal whole-blood platelet count (Mehta and Mehta, 1981). However, platelets from patients with essential hypertension do show an increased tendency to aggregate compared with normals (e.g. Someya, et al., 1984), and enhanced loss of aggregates (formed either in vivo or ex vivo) during preparation of citrated PRP would thus lead to a lower total platelet recovery. Platelets from patients with essential hypertension may be generally hyperactive, since they are known to contain elevated levels of cytosolic free Ca^{2+} (Le Quan Sang and Devynck, 1986), which is necessary for platelet shape change and aggregation (see Ardlie, 1982); also the dense granule release reaction can be induced with lower doses of thrombin than are needed for normal platelets (Valtier, et al., 1986). An enhanced release reaction would explain the decrease in serotonin levels and the elevated levels of BTG described in patients with essential hypertension (Kjeldesen, et al., 1973). In summary, the observed trends between normals and patients with essential hypertension are in agreement with those described by other groups, particularly the significant changes in PRP serotonin (e.g. Bhargava, et al., 1979; Kamal, et al., 1984a and 1984b), whereas for the other measured parameters, the changes did not reach statistical significance.

The increase in platelet serotonin in the heart failure patients receiving captopril, although not significant, may reflect a general improvement in platelet function following chronic therapy. In patients with essential hypertension, it is known that *in vitro* aggregation is diminished after captopril therapy (Someya, *et al.*, 1984) compared with normals, and this was not due to a direct effect of the drug on aggregation; it was suggested that, since captopril also inhibits the degradation of bradykinin, which in turn may lead to increased prostaglandin synthesis, the effect may be mediated through a captopril-induced increase in prostacyclin, resulting in a reduction in induced aggregation. However, as it is unlikely that prostacyclin circulates in appreciable quantities, the observed effect must be mediated by other mechanisms (see section 5.4). Pumphrey and Dawes (1982) showed that patients with heart disease had increased platelet activation *in vivo* as measured by BTG levels, so the possibility exists that in the group of heart-failure patients studied here, chronic captopril therapy has the same effect on platelet activity as has been described in patients with essential hypertension (Someya, *et al.*, 1984).

Thus in the two groups of patients with cardiovascular disease, the measured serotonin levels may reflect a) increased platelet activation in patients with untreated essential hypertension, and b) decreased platelet activation in patients with heart failure following treatment with captopril and diuretics, possibly resulting from a concomitant reduction in bradykinin degradation due to chronic captopril therapy

Chapter 7

General Discussion and Conclusions

7.1 Assays for Serotonin

The serotonin assays developed in this thesis have been used to determine serotonin status in various physiological and patho-physiological situations; the HPLC-ECD assay is simple, precise, and can be used to measure several 5-hydroxyindoleamines (or other electrochemically active compounds) in a single injection of sample; the major disadvantage with this method is that few (<20) samples can be processed within a working day, compared with up to 100/day by the RIA. The sensitivity of the HPLC-ECD assay was not studied formally, but it can be estimated by assuming that if the smallest detectable peak is 3 times the height of the baseline noise (approximately 5 mm deflection at sensitivity 0.2 nA full scale deflection), then this corresponds to a serotonin concentration of approximately 4 nmol/l, which is comparable with other published HPLC-ECD methods (Table 7.1).

A radioimmunoassay method similar to that described in this thesis has recently been published (Manz, et al., 1985). These workers raised antisera to N-succinamylserotonin-BSA after the method of Delaage and Puizillout (1981), but derivatised serotonin by acetylation with acetic anhydride; this group also monitored the acetylation by HPLC-UV, but, contrary to the results of this thesis, they found that no diacetylserotonin was produced. This conclusion was based on the observation that acetylation of serotonin followed by HPLC analysis and RIA of the fractions elution from the HPLC both yielded single peaks, but since no elution profile for diacetyl-serotonin was shown, it is possible that N-acetylserotonin and

Quoted sensitivity (nmol/l)	Reference
2.3	Fujimori, et al., 1982
<4	Petruccelli, et al., 1982
5	Tagari, et al., 1984
1	Molyneux and Clark, 1985

Table 7.1 Sensitivities of other published HPLC-ECD methods (assuming 25 µl injection).

diacetylserotonin were isographic in their HPLC system. This group did not validate their RIA with any reference method, whereas the RIA developed in this thesis is currently the only RIA for serotonin which has been validated against a reference method (HPLC-ECD). The assay has been used to measure serotonin in a variety of matrices, including human PRP, rat serum, and Medium 199 for *in vitro* platelet release studies.

The only major disadvantage with the RIA developed in this thesis is that serotonin is converted into *N*-acetylserotonin for measurement; this molecule is present in many biological matrices, and although the levels are low enough in plasma relative to serotonin to enable any interference to be ignored, this may not be the case in other biological fluids or tissues. This problem may be circumvented in a derivative assay by creation of a derivative which does not exist biologically. Such an assay has been described for histamine, where the molecule is derivatised to 2-histamyl-1,4,-benzoquinone (Guesdon, *et al.*, 1986); this would presumably work with serotonin (or any other monoamine), since the modification is carried out at pH 4.5, which would not result in the destruction of the indole nucleus.

7.2 Assay Application

Variations in sodium status in normal females significantly altered the extent of ADP induced platelet aggregation *in vitro*, PRP serotonin concentration (nmol/l), and the number of platelets recovered in PRP. There was an inverse relationship between the extent of induced aggregation, and the total serotonin concentration

(nmol/l PRP). Furthermore, salt loading resulted in a reduction in the rate and extent of ADP-induced aggregation when the serotonin antagonist ketanserin was added *in vitro*. Thus, high *in vivo* AII levels were accompanied by low levels of *in vitro* platelet aggregation; the increase in the extent of aggregation seen on salt loading may reflect an increase release of platelet constituents, possibly including serotonin. This would explain the inhibitory effect seen with the serotonin antagonist ketanserin, and also the increase in aggregation, since serotonin is known to potentiate the effect of other aggregating agents. Whether AII affects aggregation directly, or indirectly remains to be clarified, but it has been suggested that AII may stimulate the release of, or potentiate the action of TXA_2 (Ding, *et al.*, 1985b).

In the patients with heart failure, chronic captopril therapy (which is known to decrease circulating AII levels) resulted in an increase in total serotonin levels (nmol/l PRP). It is possible that this is mediated by a captopril-induced increase in bradykinin levels (the enzyme producing AII from AI also degrades bradykinin), which may result in an increased production of prostaglandins (e.g. prostacyclin), and ultimately a decrease in platelet activation. The observed increase in serotonin concentration may therefore reflect decreased platelet activation due to chronic captopril therapy in these patients.

The patients with essential hypertension had lower total serotonin (nmol/l PRP) levels than the normotensive control group. No significant difference was seen between the two groups with regard to

the kinetics of serotonin uptake. Analysis of the [^{3}H]-serotonin used for the uptake studies by HPLC-ECD showed that the purity decreases rapidly once the sealed vial is opened (<85% pure after 4 weeks, <60% pure after 16 weeks), and only the remaining radioactive serotonin is taken up into the platelets. Thus uptake values must be corrected for tracer purity, otherwise the results will be falsely depressed; this observation may partly explain reported discrepancies in the literature for absolute values for the serotonin uptake parameters, V_{max}, and K_m.

The application of the assays for serotonin in the physiological and pathophysiological studies described in this thesis show that in circumstances where platelet function increases, or is expected to increase, there is a corresponding significant decrease in the concentration of serotonin in PRP; conversely, with a decrease in platelet activity (e.g. as shown by a decrease in the extent of aggregation), there is an associated increase in PRP serotonin. These findings are in good agreement with previous similar work (Table 7.2).

It has been suggested that measurement of total plasma serotonin is a useful indicator of platelet activation *in vivo* (e.g. Parbtani, *et al*, 1980); therefore measurement of PRP serotonin concentration may be preferential to e.g. the measurement of β-thromboglobulin (BTG), since for the serotonin assay, as it is the total concentration of PRP serotonin which is measured, no special precautions need to be taken during venesection or subsequent sample processing to minimise *ex vivo* degranulation, which would otherwise

Situation	Aggregation 1	BTG 2	Total serotonin 3	References
Essential hypertension	↑	↑	↓	1: Someya, et al. 1984 2: Kjeldsen, et al., 3: Kamal, et al, 1984a & b
Heart failure	?	↑	?	1: - 2: Pumphrey and Dawes, 1982 3: -
Post myocardial infarction	↑	↑	?	1: O'Brien, et al., 1966 2: Pengo, et al., 1985 3: -
Migraine (during attack)	↑	↑	↓	1: Hilton and Cumings, 1971 2: Gawel, et al., 1979 3: Hilton and Cumings, 1972
Diabetes	↑	↑	?	1: Kubisz, et al., 1984 2: Burrows, et al., 1978 3: -
Sodium restriction	↓	?	*↑	1: Nara, et al., 198 2: - 3: -

Table 7.2 The relationship between the extent of induced platelet aggregation, in vivo platelet activation as measured by BTG levels, and total plasma serotonin levels in various pathological or non-pathological situations.

* = result obtained in PRP in this thesis
? = as yet unknown

result in falsely elevated values with BTG assays.

In the studies carried out in this thesis, although significant differences were seen when comparing the levels of serotonin in PRP between two groups, these differences were not significant after correcting for platelet numbers, indicating differences in platelet counts in the groups studied. Figure 7.1a shows the correlation obtained by linear regression analysis of PRP serotonin levels (nmol/l), and the corresponding values after correcting for the PRP platelet number for 162 consecutive samples from patients and normal volunteers (nmol/l = 4.67 x pmol/10^8 platelets - 14.2; n = 162; r = 0.917); Figure 7.1b shows the correlation obtained by linear regression analysis of the natural logarithm of both sets of data (ln nmol/l = 0.865 x ln pmol/10^8 platelets + 1.0; n = 162; r = 0.833). In each case, the slope of the line is significantly different from zero ($p<0.0001$ in both instances). These data suggest that when compared with platelet serotonin concentration (i.e. pmol/10^8 platelets), measurement of PRP serotonin (i.e. nmol/l PRP) is an equally reliable index of physiological changes in blood serotonin concentration, suggesting that the significant differences found in this thesis when comparing PRP serotonin levels between two groups are real.

7.3 Future Prospects

As outlined above, the results obtained in this thesis and work from other groups (Nara, et al., 1984; Ashida, et al., 1985) suggest that a change in sodium status affects platelet activation. If this

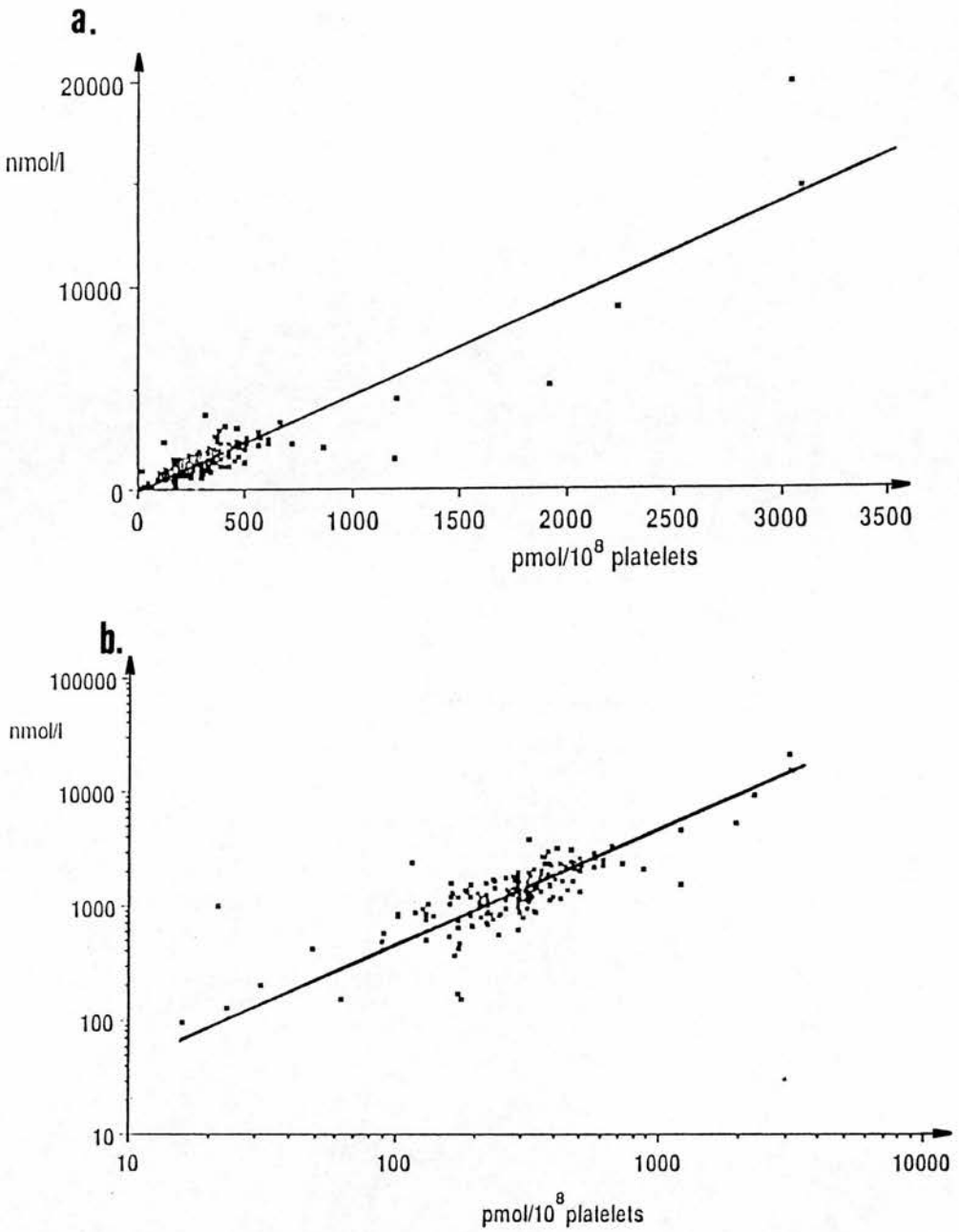


Figure 7.1 Correlations between

a) serotonin (pmol/10^8 platelets) and serotonin (nmol/l PRP), and

b) serotonin (ln pmol/10^8 platelets) and serotonin (ln nmol/l PRP).

relationship is partly mediated by an increase in the release of serotonin from platelets during sodium load, it should be possible to detect both an increase in the release reaction itself *in vitro* and at the same time an increase in the circulating levels of the indicator of platelet release, BTG. If an enhanced release of platelet constituents does occur during salt loading, then it is anticipated that there would be an inverse correlation between PRP serotonin concentration and the concentration of BTG present in PPP. The release of serotonin from platelets can be measured directly by the RIA developed in this thesis, obviating the need to pre-incubate platelets with either [^{3}H]- or [^{14}C]-serotonin, and subsequent measurement of released serotonin by liquid scintillation counting.

Further study is required concerning the modulation of circulating AII levels by ACE inhibition; although published work (Someya, *et al.*, 1984) and the results presented in this thesis indicate that there may be a decrease in platelet activity following captopril therapy, it remains unclear how this is brought about. As discussed earlier (section 5.6), it has been suggested that ACE inhibition may result in elevation of prostacyclin due to an increase in bradykinin. Consequently, blockade of prostaglandin synthesis e.g. by indomethacin or aspirin, with and without concomitant ACE inhibition may help to indicate how prostaglandins contribute to the observed effects, thus leading to a better understanding of the role of serotonin and the platelet in vascular diseases.

Chapter 8

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Appendix I

Appendix I Program to Fit Line Using Revised Direct Linear Plot

```
10 CLS
20 REM DISTRIBUTION FREE ANALYSIS
30 REM IDENTIFICATION!
40 INPUT "     PATIENT NAME";P$
50 INPUT " EXPERIMENT DATE";E$
60 PRINT:PRINT:PRINT:PRINT
70 PRINT " PATIENT:-",P$
80 PRINT "     DATE:-",E$
90 PRINT:PRINT:PRINT:PRINT
100 DIM GRAD(50),X(10),Y(10),INTERCEPT(50)
110 REM INPUT NUMBER OF DATA POINTS TO BE HANDLED
120 INPUT " NUMBER OF DATA POINTS";POINTS
130 REM CALCULATE NUMBER OF LINES JOINING ALL POINTS
140 LINES =.5*POINTS*(POINTS-1)
150 REM INPUT DATA
160 FOR COUNT=1 TO POINTS:INPUT " X VALUE";X:X(COUNT)=X
170 INPUT " Y VALUE";Y:Y(COUNT)=Y
180 NEXT COUNT
190 LPRINT,," PATIENT:-",P$
200 LPRINT,,"     DATE:-",E$
210 LPRINT:LPRINT:LPRINT
220 FOR COUNT = 1 TO POINTS
230 LPRINT "           X "COUNT" = ";:LPRINT USING "####.###";X(COUNT);
240 LPRINT "           Y "COUNT" = ";:LPRINT USING "####.###";Y(COUNT)
250 NEXT COUNT
260 A=1:T=1
270 REM CALCULATE GRADIENTS
280 FOR C=A TO (POINTS-1)
290 REM NO DIVISION BY ZERO!!!!!
300 IF X(A)=X(C+1) THEN GRAD(T)=9999:INTERCEPT(T)=9999:GOTO 340
310 GRAD(T)=((Y(A)-Y(C+1))/(X(A)-X(C+1)))
320 INTERCEPT(T)=(X(A)*Y(C+1)-X(C+1)*Y(A))/(X(A)-X(C+1))
330 IF INTERCEPT(T)<0 THEN INTERCEPT(T)=.0001:GRAD(T)=1
340 T=T+1:NEXT C:A=A+1
350 REM CHECK ALL LINES DONE
360 IF T>LINES THEN GOTO 390
370 REM IF NOT, THEN BACK TO LOOP
380 GOTO 280
390 REM PRINT RESULTS
400 PRINT:PRINT:PRINT
410 LPRINT:LPRINT:LPRINT:LPRINT
420 FOR T=1 TO LINES
430 IF GRAD(T)=9999 THEN PRINT " GRADIENT =    INFINITE ";
440 IF GRAD(T)=9999 THEN LPRINT ," GRADIENT =    INFINITE ";:GOTO 470
450 PRINT " GRADIENT = ",:PRINT USING "####.###";GRAD(T),
460 LPRINT ," GRADIENT = ",:LPRINT USING "####.###";GRAD(T),
470 IF INTERCEPT (T)=9999 THEN PRINT "     INTERCEPT =          NONE"
480 IF INTERCEPT (T)=9999 THEN LPRINT "      INTERCEPT =          NONE":GOTO 510
490 PRINT "      INTERCEPT = ",:PRINT USING "####.###";INTERCEPT(T)
500 LPRINT "      INTERCEPT = ",:LPRINT USING "####.###";INTERCEPT(T)
510 NEXT T
520 REM SORT ROUTINE
530 FOR N=LINES TO 1 STEP -1
540 FOR A=1 TO (N-1)
550 IF GRAD(A)>GRAD(A+1) THEN B=GRAD(A):GRAD(A)=GRAD(A+1):GRAD(A+1)=B
560 IF INTERCEPT(A)>INTERCEPT(A+1) THEN B=INTERCEPT(A):INTERCEPT (A)=INTERCEPT(A
+1):INTERCEPT (A+1)=B
570 NEXT A
580 NEXT N
590 PRINT:PRINT:PRINT
600 LPRINT:LPRINT:LPRINT
610 PRINT," RESULTS ARRRANGED IN ASCENDING ORDER:-"
620 LPRINT," RESULTS ARRRANGED IN ASCENDING ORDER:-"
630 PRINT:PRINT
640 LPRINT:LPRINT
650 PRINT:PRINT:PRINT:PRINT
660 LPRINT ,"                GRADIENTS",,"INTERCEPTS"
```

```
670 FOR COUNT=1 TO LINES
680 PRINT,:PRINT USING "####.###";GRAD(COUNT),
690 LPRINT,,:LPRINT USING "####.###";GRAD(COUNT),
700 PRINT,,;:PRINT USING "####.###";INTERCEPT(COUNT)
710 LPRINT,,;:LPRINT USING "####.###";INTERCEPT(COUNT)
720 NEXT COUNT
730 MEAN=LINES/2
740 REM CALCULATE IF LINES IS ODD OR EVEVN
750 IF MEAN-CINT(MEAN)=0 THEN GOTO 820
760 REM MEDIANS FOR ODD NUMBER OF LINES
770 PRINT:PRINT:PRINT
780 LPRINT:LPRINT:LPRINT
790 G=((LINES-1)/2)+1:G=GRAD(G)
800 I=((LINES-1)/2)+1:I=INTERCEPT(I)
810 GOTO 870
820 REM MEDIANS FOR EVEN NUMBER OF LINES
830 PRINT:PRINT:PRINT
840 LPRINT:LPRINT:LPRINT
850 G=(GRAD(LINES/2)+GRAD((LINES/2)+1))*.5
860 I=(INTERCEPT(LINES/2)+INTERCEPT((LINES/2)+1))*.5
870 PRINT," MEDIAN GRADIENT  =";:PRINT USING"####.###";G
880 LPRINT,," MEDIAN GRADIENT  =";:LPRINT USING"####.###";G
890 PRINT," MEDIAN INTERCEPT =";:PRINT USING"####.###";I
900 LPRINT,," MEDIAN INTERCEPT =";:LPRINT USING"####.###";I
910 PRINT:PRINT
920 LPRINT:LPRINT
930 PRINT,"             V MAX =";:PRINT USING"####.###";100/I
940 LPRINT,,"             V MAX =";:LPRINT USING"####.###";100/I
950 PRINT,"             K M   =";:PRINT USING"####.###";1000*G/I
960 LPRINT,,"             K M   =";:LPRINT USING"####.###";1000*G/I
970 FOR P=0 TO 9:LPRINT:NEXT P
980 END
```

Appendix II

Appendix II New York Heart Association Classification

Class I - **No limitation.** Ordinary physical activity does not cause undue fatigue, dyspnoea, or palpitation.

Class II - **Slight limitation of physical activity.** Comfortable at rest. Ordinary physical activities result in fatigue, palpitations, dyspnoea, or angina.

Class III - **Marked limitation of physical activity.** Comfortable at rest. Less than ordinary activity will lead to symptoms described in Class II.

Class IV - **Inability to carry out any physical activity without discomfort.** Symptoms of congestive cardiac failure present at rest, increased discomfort with any physical activity.

Appendix III

Appendix III Preparation of N-Acetoxysuccinimide

"...N-Acetoxysuccinimide. To N-hydroxysuccinimide (5 g, 43.5 mmol) in 150 ml of acetone was added 4.1 ml (43.5 mmol) of acetic anhydride dropwise at room temperature with stirring. After 160 min the solvent was evaporated under vacuum, the residue redissolved in 150 ml of absolute ethanol at 45°C, and the solution cooled to 4°C, yielding 5.39 g (88% yield) of product, mp 132-133°C (lit. mp 132-133°C (Baumann et al., 1973))..."

Treadway and Schultz, 1976

Appendix IV

Appendix IV Short Protocols

1. HPLC-ECD Serotonin Assay

To the sample (400 µl), add the internal standard (50 µl, 25 µmol/l in saline), vortex, add 50 µl of a solution of perchloric acid/cysteine (1.5 mol/l and 2 mmol/l respectively), vortex again, and leave at least 15 min at 4°C. Sample is then centrifuged at 4°C for at least 15 min at 1,720 x g (or greater) to pellet precipitated protein. Sample can then be injected directly, or removed to fresh tube and stored at 4°C for at least 6 h.

2. RIA for Serotonin in Human PRP

Sample is diluted 1/20 in assay buffer; standards (1 - 1,000 nmol/l) are made up in PPP diluted 1/20 in assay buffer (PPP must contain <20 nmol/l serotonin). To the sample/standard (450 µl) add 50 µl perchloric acid/cysteine (1.5 mol/l and 2 mmol/l respectively), and treat as above. Take duplicate aliquots (2 x 200 µl) of protein-free supernatants, and add to N-acetoxysuccinimide (250 µg, dried down from a 5 mg/ml methanolic stock) in glass tubes. Vortex, and add 50 µl of KOH or NaOH (37.5 µmol) in 0.5 mol/l protein-free assay buffer (original pH 6.2). Vortex, and leave for 60 min at room temperature; add glycine (8.3 µmol) in assay buffer (50 µl), vortex, and leave 30 min. Add tracer in assay buffer (100 µl), followed by a freshly prepared mixture of NIRS, DARS, and primary antiserum in assay buffer (300 µl). Vortex and incubate overnight at 4°C. Separate by adding 1 ml cold (4°C) buffer prewash, and centrifuge at 4°C and 1,720 x g for 20 min. Aspirate, and count pellet (bound fraction).

Appendix V

Appendix V Miscellaneous Precision Data

Assay	Intra-assay %CV
Aldosterone	< 8
Angiotensin II	<15
Na^+/K^+	< 2

Only the intra-assay percentage coefficient of variation (%CV) is given, since the samples from the studies which used the above assays were run within a single assay.

Appendix VI

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Clinica Chimica Acta, 162 (1987) 175–188
Elsevier

CCA 03678

Development and validation of an improved radioimmunoassay for serotonin in platelet-rich plasma

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(Received 25 March 1986; revision received 19 September 1986; accepted after revision 20 September 1986)

Key words: Serotonin; Radioimmunoassay; HPLC; *N*-Acetylation

Summary

A radioimmunoassay (RIA) using a ^{125}I-tracer is described for measurement of serotonin (5-hydroxytryptamine, 5-HT) in human platelet-rich plasma (PRP). Antisera were raised against 5-HT-succinamate conjugated to bovine albumin and, to improve assay sensitivity, the analyte was made chemically similar to the immunogen by conversion to *N*-acetylserotonin prior to assay, using the specific amino reagent *N*-acetoxysuccinimide. The assay shows good correlation with a high-pressure liquid chromatography (HPLC) reference method ($5\text{-HT}_{RIA} = 1.007 \times 5\text{-HT}_{HPLC} + 29.3$, $r = 0.936$, $p < 0.001$, $n = 40$), indicating that no significant cross-reactions were detected. Samples of PRP are diluted 1/20 to fall within the working range (80–15% B/B_0) of the assay, which is 4.75–325 nmol/l, (0.95–65.0 pmol/tube), corresponding to 95–6500 nmol/l in PRP. Intra- and interassay coefficients of variation were 5.0–10.5% and 12.0–21.2% respectively for serotonin concentrations of 250–2 500 nmol/l added to platelet-poor plasma. With this improved assay, it is possible to analyse up to 100 samples/day, compared with 10–20 samples/day by HPLC.

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Introduction

Abnormal concentrations of serotonin (5-hydroxytryptamine, 5-HT) in platelets have been described in several pathological conditions, including essential hypertension [1–3], migraine [4,5], depression [6], carcinoid syndrome [7], liver cirrhosis [8], and pre-menstrual tension [9]. Despite this growing body of evidence implicating serotonin as a possible biochemical marker in disease, the development and validation of an assay capable of handling the large numbers of samples necessary for comprehensive studies has been slow.

Serotonin is conventionally measured by high-pressure liquid chromatography (HPLC) with electrochemical detection [10–12], radioenzymatic assay [13–16], or radioimmunoassay (RIA, [17–21]). HPLC has the advantage that 5-HT, its precursors and metabolites can be measured in the same injection, but few samples (< 20) can readily be assayed in a working day. Radioenzymatic assays are multi-step procedures, and ultimately involve extraction of a tritiated product followed by liquid scintillation counting. Of the existing RIA methods for serotonin only two use gamma-labelled tracers [20,21], which would make them more suitable for use as a routine assay. The remainder use [^{3}H]serotonin as the tracer, and low working dilutions of rabbit antisera (typically 1/300), which show high cross-reactivity with 5-methoxytryptamine [17–19], although since the level of this in plasma is normally low [19], interference would only be significant if 'free' (i.e. non-platelet bound) concentrations of serotonin were being measured. Since rabbit serum contains a high level of serotonin (approximately 20 μmol/l [22]), this must be removed to avoid a decrease in sensitivity in the RIAs using [^{3}H]serotonin as tracer. This can be achieved either by dialysis of the antiserum [18], or by collecting plasma instead of serum [19] and rendering it platelet-free by centrifugation (almost all serotonin in blood is in platelets [23], and may be released during clotting).

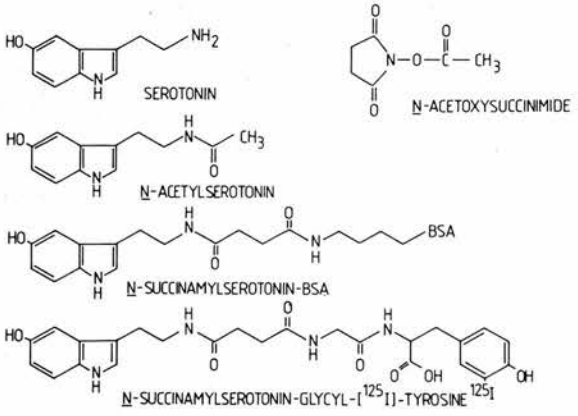

Fig. 1. Structure of serotonin, its acylated derivatives, and the acetylating agent *N*-acetoxysuccinimide.

The assays with gamma-labelled tracers [20,21] use antibodies raised to either *N*-succinamylserotonin or *N*-succinamyl-5-methoxytryptamine and avoid a loss in sensitivity by chemically modifying the analyte into the *N*-succinamyl form [20], or to *N*-acetyl-5-methoxytryptamine (melatonin [21]) prior to assay. Consequently, unmodified serotonin is poorly recognised in these systems which avoids the need to remove endogenous antigen from the antisera. A disadvantage with the first method is that the succinylating agent used (succinic anhydride) reacts with 5-HT both at the amino and the 5-hydroxyl group and the 5-*O*-hemisuccinyl moiety must then be hydrolysed by alkali [24] to render the molecule fully immunoreactive. In the second method, the 5-hydroxyl group is chemically methylated, and is therefore protected from reaction with the acetylating agent (acetic anhydride). This assay still requires a final organic extraction step, but it demonstrates that the chemically similar *N*-acetyl derivative (Fig. 1) can be recognised by an antiserum raised against the *N*-succinamyl derivative. A similar approach has been described for cyclic nucleotide assays [25]. Potential interference by endogenous *N*-acetylserotonin was not fully considered in the original papers [20,21], and the assays were not validated by a reference method. We have developed and validated by HPLC a radioimmunoassay which broadly follows a published method [20], but which incorporates certain improved features.

Materials and equipment

Serotonin creatinine sulphate, *N*-acetylserotonin, tryptamine, tryptophan, 5-hydroxytryptophan, 5-hydroxyindoleacetic acid, melatonin, cysteine hydrochloride, isobutyl chloroformate and Amberlite XAD-2 were all purchased from Sigma, Poole, Dorset, UK. Bovine serum albumin (BSA), dimethylformamide and dioxan were from BDH, Poole, Dorset, UK. The perchloric acid was from May and Baker, Dagenham, Essex, UK, the *N*-methylserotonin and tri-*n*-butylamine were from Aldrich, Gillingham, Dorset, UK. Tritiated serotonin was from Amersham International, Amersham, Bucks, UK, and Sephadex G-50 was from Pharmacia, Milton Keynes, Bucks, UK. Donkey anti-rabbit serum, and non-immune rabbit serum were from the Scottish Antibody Production Unit, Law Hospital, Carluke, UK. Assay buffer, pH 6.2, contained 0.1 mol/l citric acid, 0.3 mol/l sodium hydroxide, 1 mmol/l EDTA, and 1 g/l gelatin. The multi-well gamma counter with software was supplied by Nuclear Enterprises, Sighthill, Edinburgh, UK. Glass assay tubes (12×75 mm) were from Corning, New York, NY, USA, and polypropylene microfuge tubes were from Macfarlane Robson, Glasgow, Scotland, UK. The HPLC equipment (Waters/Millipore, Harrow, Middlesex, UK) comprised an M6000A pump and U6K injector with a 2-ml sample loop, a precolumn (3.9 mm $\times$ 2 cm) packed with C_{18} Corasil (37–50 μm diameter), and either a Waters 3.9 mm $\times$ 30 cm C_{18} μBondapak column (particle diameter 10 μm), or a Capital HPLC (Edinburgh, UK) column (4.9 mm $\times$ 10 cm) packed with Hypersil ODS (particle diameter 5 μm). Detectors used were a Bioanalytical Systems (West Lafayette, NJ, USA) LC-4A electrochemical detector with a single glassy carbon electrode, or a Waters 441 UV detector. Peaks were plotted on a Waters data module, and peak heights used for

calculation of concentrations. Mobile phase was de-gassed with a Waters solvent clarification kit, through a Millipore 'Durapore' filter (0.22 μm). Methanol and water for HPLC were from Rathburn Chemicals, Walkerburn, Peebleshire, UK.

Non-HPLC organic solvents were redistilled prior to use. The remaining chemicals were all of analytical grade.

Methods

Standards

Standard serotonin (serotonin creatinine sulphate) was stored as a concentrated (4–5 mmol/l) stock dissolved in 0.15 mol/l perchloric acid/0.2 mmol/l cysteine (pH 1.8); *N*-acetylserotonin was dissolved first in ethanol (approximately 500 μl), then diluted in acid/cysteine; both were then stored at 4°C in the dark. Concentrations were checked by UV absorption at 275 nm for serotonin, and 278 nm for *N*-acetylserotonin (the molar extinction coefficient, ϵ, had previously been determined to be 5740 l/mol per cm for serotonin, and was assumed to be the same for *N*-acetylserotonin).

Preparation of platelet-rich and platelet-poor plasma (PRP and PPP)

Whole blood was taken from the antecubital vein using a 19 gauge needle with tourniquet, added to the anticoagulant (citric acid (8 g/l), trisodium citrate (22 g/l), glucose (20 g/l) in 10 ml plain polystyrene tubes; 9 vol blood : 1 vol anticoagulant) and used to prepare either PRP or PPP.

PRP The blood was centrifuged immediately at room temperature for 10 min at $121 \times g$, and the upper three-quarters of the supernatant (PRP) harvested with a disposable transfer pipette and stored frozen at −20°C.

PPP The blood was centrifuged immediately at 4°C for 1 h at $1\,720 \times g$, and, to avoid inclusion of platelets trapped in the floating lipid layer, or from the plasma/red cell interface, the middle third of the supernatant (PPP) was removed and stored frozen at −20°C. The serotonin content of the PPP was measured by the HPLC assay, and was only used for the RIA if the concentration was < 20 nmol/l, as this would be undetectable after the 20-fold dilution step required for the RIA.

Deproteinisation

Preparation of samples for assay by HPLC

Samples of PRP (400 μl) previously frozen and thawed to rupture the platelets were spiked with 1.25 nmol of the internal standard *N*-methylserotonin [26] in assay buffer (50 μl), then deproteinised.

Preparation of samples for RIA

Samples of PRP or standards diluted in PPP were diluted 20-fold in assay buffer, and aliquots (450 μl) deproteinised.

Deproteinisation

The prepared sample/standard (450 μl) was dispensed into a polypropylene conical microcentrifuge tube (1.5 ml), and the proteins precipitated by adding an aliquot (50 μl) of perchloric acid/cysteine solution (1.5 mol/l and 2 mmol/l, respectively). The tube was vortexed, left at 4°C for 15 min, and then centrifuged at $10\,000 \times g$ for 15 min at 4°C. The supernatant was removed to a clean polypropylene tube and stored at 4°C until assayed, which must be within the same day.

HPLC assay

The mobile phase (pH 4.8) contained water (1 700 ml), methanol (300 ml), sodium dihydrogen orthophosphate (10.8 g), ethylenediamine tetra-acetic acid disodium salt (400 mg), and sodium 1-octanesulphonate (240 mg), and was degassed before use. Flow rates were 0.5–1.0 ml/min, and the detector was set at +0.70 V with 5 nA full scale deflection. Standards were diluted from the concentrated stock into perchloric acid/cysteine (0.15 mol/l and 0.2 mmol/l, respectively) each assay day, and stored in the dark at 4°C throughout the assay run. Standards and deproteinised supernatant from samples were injected directly (20–100 μl). This system separated the major 5-HT metabolite 5-hydroxyindoleacetic acid (5-HIAA), *N*-acetylserotonin, serotonin, and the internal standard, *N*-methylserotonin.

Radioimmunoassay

Synthesis of N-succinamylserotonin and immunogen

N-Succinamylserotonin was synthesised and conjugated to bovine serum albumin by methods modified from those of Delaage and Puizillout [20].

Serotonin creatinine sulphate (61 mg) and succinic anhydride (91 mg) were stirred under nitrogen for 10 min with 0.68 mol/l potassium hydroxide (2.16 ml). A solution of hydroxylamine hydrochloride (21 mg) in water (0.5 ml) was added, followed by 0.68 mol/l potassium hydroxide (1.55 ml), and the solution was stirred for a further 5 min, then adjusted to pH 4 by addition of 1 mol/l hydrochloric acid. The solution was rapidly applied to a column of Amberlite XAD-2 (25 ml; previously freed of UV-absorbing contaminants by exhaustive washing with ethanol) in water, and the column was then washed with water (approximately 200 ml) until the eluate showed no UV absorption. The column was eluted with ethanol (150 ml), and the yield of *N*-succinamylserotonin determined by its UV absorption at 278 nm ($\epsilon_{278} = 5740$ l/mol per cm).

The ethanolic solution was evaporated under reduced pressure, and the residue redissolved in water (10 ml), and lyophilised to leave the product as a pale foam, which was either used directly for conjugation to bovine serum albumin, or redissolved in dry dimethylformamide (DMF) at 17.8 mg/ml. Aliquots of this solution were sealed under nitrogen in glass ampoules to avoid oxidation and stored at −20°C for use in the preparation of the radioiodinated tracer.

For the preparation of the immunogen, a solution of *N*-succinamylserotonin (38 mg) in dry dioxan (2.14 ml) was cooled to 10–12°C, and treated with tri-*n*-butylamine (62 μl), and isobutyl chloroformate (15 μl). After 20 min this solution was

added under nitrogen to an ice-cold solution of bovine serum albumin (332 mg) in water (8.8 ml), 1 mol/l sodium hydroxide (0.33 ml) and dioxan (5.7 ml). This solution was stirred in an ice bath for 1 h, then treated with 0.25 mol/l sodium phosphate, pH 6 (1.4 ml), and dialysed for 1 h against 0.05 mol/l sodium phosphate, pH 6 (5 l). The dialysate was subjected to gel filtration on Sephadex G-50 (40 × 3 cm), eluting with 0.05 mol/l sodium phosphate, pH 6. The fraction eluted in the void volume was analysed by UV spectroscopy and Lowry protein estimation, which showed a conjugation ratio of 24 serotonin residues per protein molecule. The eluate was lyophilised and the conjugate stored at −20°C.

Immunisation and bleeding

Four rabbits (New Zealand White) were used, and immunisation was at 4 sites sub-cutaneously throughout. The dose was 100 μg/animal in each case, in 1 : 1 Freund's complete adjuvant : saline for the primary, and in 1 : 1 Freund's incomplete adjuvant : saline for the boosts. Boosts were given 6 wk after the primary immunisation, and thereafter at intervals of 6 wk. Bleeds were taken 10 days after each boosting. Blood was collected as heparinised PPP for the early bleeds and as serum from later bleeds. This enabled larger volumes of blood to be collected, and had no effect on titre. The plasma/serum was stored at −20°C either neat, or as 1/10 dilution in assay buffer.

Radioiodination

The procedure was similar to that already described [20]. All solutions were freshly prepared on the day of the iodination. An aliquot (50 μl) of *N*-succinamylserotonin in DMF (17.8 mg/ml) was taken from one of the sealed vials, and dispensed into a conical polypropylene microcentrifuge tube. To this was added a solution (10 μl) of tri-*n*-butylamine in DMF (17 μl/ml), followed by a solution (10 μl) of isobutyl chloroformate diluted in DMF (45 μl/8.5 ml). The tube was vortexed and incubated for 20 min at 10–12°C. Glycyl tyrosine (476 ng) in 0.25 mol/l, pH 7.5, phosphate buffer (10 μl) was added to a 1 mCi solution (10 μl) of $Na^{125}I$ (spec act, 13–17 mCi/μg I) and mixed. Chloramine-T (10 μg) dissolved in the same phosphate buffer (10 μl) was added, the solution mixed, and left at room temperature for 1 min. The reaction was stopped by the addition of sodium metabisulphite (12 μg) in 0.05 mol/l phosphate buffer, pH 7.5 (20 μl). The activated *N*-succinamylserotonin solution (50 μl) was added to the iodinated glycyl tyrosine, the solutions mixed, and then left at 4°C for 5 min. The reaction mixture was diluted with 500 μl of the eluting buffer (0.1 mol/l citric acid, 0.1 mol/l sodium chloride, 3 mmol/l ascorbic acid, final pH 6.2) before being applied to the Sephadex G-25 column (49 × 1 cm). The buffer was pumped through the column at 1 ml/min, and fractions (6 ml) collected in plain polystyrene tubes. Aliquots (10 μl) of the fractions were counted in a multiwell gamma-counter to locate the peaks. The immunoreactive fractions of a single peak (typically fractions 25–30) were pooled, diluted 1 + 1 with assay buffer, and stored at −20°C as aliquots (2 ml) in stoppered polystyrene tubes. Each aliquot was thawed once only, after which it was stored in the dark at 4°C and discarded after 1 wk.

Testing of antisera

Antisera were tested by incubation of varying dilutions of primary antiserum (100 μl) with a fixed mass (1 fmol, 5000 dpm) of tracer in buffer (100 μl), and solutions of 30-fold diluted donkey anti-rabbit serum (100 μl) and 300-fold diluted normal rabbit serum (100 μl). Incubation was overnight at 4°C, and prior to separation, a prewash (1 ml) of cold (4°C) assay buffer was added, and the tubes centrifuged in pre-cooled carriers at 1720 × g for 30 min at 4°C. The supernatants were aspirated, and the pellet (bound fraction) counted in a multi-well gamma-counter.

Acetylation

The acetylating agent *N*-acetoxysuccinimide (NAS) was prepared according to the method of Treadway and Schultz [27]. For each assay, a fresh solution of NAS in methanol (5 mg/ml) was prepared, and aliquots (50 μl) dispensed into glass tubes. The methanol was evaporated under a stream of nitrogen whilst warming the tubes (45°C). Acidified deproteinised supernatants from standards or samples were taken in duplicate (each 200 μl) and added to the dried NAS followed by 37.5 μmol sodium hydroxide in protein-free 0.5 mol/l citrate assay buffer (50 μl). The tubes were vortexed, then left 1 h at room temperature. Glycine (8.3 μmol) was added in assay buffer (50 μl), the tubes vortexed, and left a further 30 min at room temperature. If no acetylation was required (e.g. in some of the cross-reactivity studies), NAS was omitted from the methanol.

Assay procedure

Samples of PRP or standards in PPP were deproteinised and acetylated. Tracer (1 fmol) diluted in assay buffer (100 μl) was added, followed by a pre-mix (300 μl) of primary antiserum (rabbit 16 bleed 4, R16B4), donkey anti-rabbit antiserum, and normal rabbit serum, at final dilutions of 1/28000, 1/210, and 1/2100, respectively, in the final incubate (700 μl). The tubes were vortexed, incubated overnight at 4°C, and separated as described above. Calculation of results was by software supplied with the multi-well gamma-counter, using a regression plot of logit % B/B_0 versus log concentration.

Cross-reactivities

These studies were performed with the test compounds and serotonin diluted in assay buffer prior to deproteinisation. Cross-reactions were measured for the test compounds themselves and where appropriate, after *N*-acetylation as above. Percentage cross-reactivity was calculated from the ratio of the mass *N*-acetylserotonin at 50% B/B_0: mass of cross-reactant at 50% B/B_0.

Precision studies

Serotonin was added to PPP from normal subjects at concentrations of 250, 750, and 2500 nmol/l, and aliquots (1 ml) frozen at −20°C in polypropylene micro-centrifuge tubes. These recovery pools were assayed by HPLC and RIA to determine intra- and inter-assay precision over a period of 8 wk.

Validation of RIA by HPLC

Samples of PRP from normal volunteers or from patients were analysed by both HPLC and RIA, and the results correlated by linear regression analysis.

Results

Stability of standards

Serotonin was found to be acceptably stable when stored as described. When compared by HPLC analysis with a freshly prepared serotonin standard, a solution which had been stored and used over a period of 9 mth was found to contain 93.0% of the original serotonin concentration.

Recovery after deproteinisation

The recovery after deproteinisation of tritiated serotonin (> 90% pure) from undiluted PRP was (mean ± SD) 92.0 ± 2.9% for samples of the same plasma ($n = 7$), and 91.5 ± 1.8% for plasma from different volunteers ($n = 7$). There was no significant difference between the two recoveries as calculated by the Student's t test ($p > 0.5$).

With the HPLC internal standard, recoveries from samples of the same or different plasmas were 80.4 ± 3.7% ($n = 7$), and 79.1 ± 2.7% ($n = 7$), respectively. Again, there was no significant difference between the two. However the recovery of [^{3}H]serotonin was always significantly greater than the internal standard recoveries ($p < 0.001$, Student's t test).

Testing of antisera

All rabbits used gave antisera which bound the tracer and could be displaced by authentic *N*-acetylserotonin. Titres (the dilutions of antisera binding 50% of the tracer in the absence of cold antigen) from the first bleeds were all greater than 1/2 500, and in one rabbit (R16) rose to a maximum (1/56 000) at bleed 6. No change in sensitivity was seen in R16 antisera despite the rising titre. Of the rabbits and bleeds tested, one antiserum (R16B4) was selected for use on the basis of good titre coupled with good sensitivity.

Radioiodination

Incorporation of ^{125}I into *N*-succinamylserotonin–glycyl tyrosine was 28.3 ± 8.5% (mean ± SD, $n = 5$). On incubation with excess antiserum, 80–90% of the tracer could be recovered in the bound fraction and non-specific binding was 2–4%. The specific activity as calculated from depression of zero binding associated with increasing tracer mass was found to be 2 011 Ci/mmol. The tracer showed no loss in immunoreactivity over a period of 5 mth stored in aliquots at −20°C, if the mass of tracer added per tube was kept constant.

N-Acetylation

Preliminary experiments had shown that at pH 6, 250 μg of NAS could acetylate 85–90% of a 3 μmol/l solution of serotonin in deproteinised PPP (200 μl). This

mass of NAS was therefore used for all subsequent acetylations. The acetylation reaction was essentially complete after 40 min, but was routinely left a further 20 min. No degradation of the acetylated product was seen even after 160 min at room temperature. The optimum pH for acetylation was found to be > 5, and the reaction was almost completely inhibited below pH 3. The added glycine was intended to react with any remaining NAS, which may otherwise have acetylated the antibody and impaired immunoreactivity.

Radioimmunoassay

A typical standard curve is shown in Fig. 2, with associated precision profile [28]. The formal sensitivity of the standard curve (2.5 SD from the zero standard) was 2.0 nmol/l or 0.40 pmol/tube (70.4 pg/tube), which, corrected for dilution, corresponds to a serotonin concentration in PRP of 40 nmol/l. As precision at the formal detection limit is generally poor for RIAs, we have limited the working range of the assay to 80–15% B/B_0, which corresponds to 4.75–325 nmol/l for a sample of 200 μl, or 0.95–65 pmol/tube (0.17–11.44 ng/tube). This represents concentrations in undiluted PRP of 95–6 500 nmol/l. Addition of the antisera as a preformed first/second antibody complex had no effect on the characteristics of the standard curve as compared with a 4 h primary incubation followed by addition and overnight incubation with the double antibody reagents. Changing the buffer from pH 6.2 to pH 4 lowered the zero binding from 60% to 53%. The overall recovery of serotonin (deproteinisation recovery of 80%, acetylation efficiency 85%) was approximately 68%.

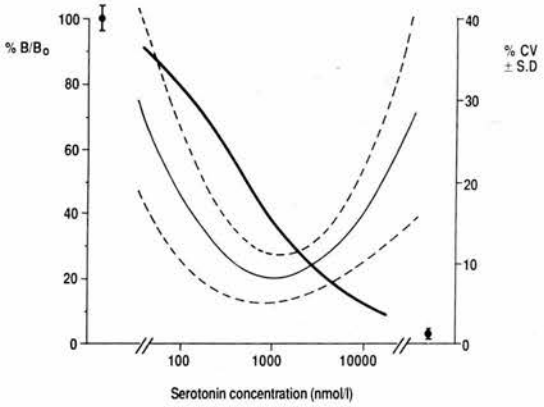

Fig. 2. Standard serotonin curve (mean value, ———), and precision profile (mean value, ———, ± SD, - - - - - -) for eight consecutive assays showing intra-assay variation of duplicate mean estimations. Primary antiserum dilution was 1/28000 (final) added as a pre-formed first antibody/second antibody/carrier complex. With an added tracer mass of 1 fmol and an overnight incubation at 4°C, zero binding (B_0) was 48.8 ± 6.1% of the total added radioactivity.

TABLE I

Cross-reactivities of the R16 antiserum

Compound	% Cross-reactivity
N-Acetylserotonin	100.00
Serotonin	0.12
5-Hydroxyindoleacetic acid	< 0.03
N-Acetyltryptamine	0.03
N-Acetyl-5-methoxytryptamine	< 0.02
N-Acetyl-5-hydroxytryptophan	< 0.01
5-Hydroxytryptophan	< 0.01
Tryptamine	< 0.01

Cross-reactivities

The major (0.12%) formal cross-reactant (see Table I) was unacetylated serotonin, but this would not normally be present in assay incubates. *N*-Acetylation increased the cross-reactivity of the other indoleamines, but methylation of the 5-hydroxyl group of *N*-acetylserotonin reduced cross-reactivity from 100% to < 0.02%.

Intra- and inter-assay variation

Intra- and inter-assay variation (Table II) in the HPLC assay were lower than in the RIA at all the tested concentrations, but the recoveries of serotonin from spiked PPP were very similar for both assays. No decrease in the values obtained from the spiked pools was seen over a period of 2 months (Fig. 3).

TABLE II

Intra- and inter-assay variation for RIA and HPLC assay

	PPP spiked with 5HT (nmol/l)	HPLC		RIA	
		$\bar{x} \pm$ SD	%CV	$\bar{x} \pm$ SD	%CV
Intra-	250	252.0 ± 5.2	2.1	214.8 ± 22.5	10.5
assay	750	884.2 ± 5.7	0.6	773.6 ± 79.6	10.4
variation	2500	2579.0 ± 79.1	3.1	2296.8 ± 131.1	5.7
n =		5		10	
Inter-	250	259.3 ± 14.8	5.7	242.4 ± 36.6	21.2
assay	750	836.9 ± 46.9	5.6	885.0 ± 106.6	12.0
variation	2500	2432.0 ± 158.8	8.2	2465.5 ± 364.8	14.8
n =		12		12	
Inter-	250	103.8 ± 5.9	–	97.0 ± 20.6	–
assay	750	111.6 ± 6.2	–	118.0 ± 14.2	–
% recovery	2500	97.3 ± 8.0	–	98.6 ± 14.6	–
n =		12		12	

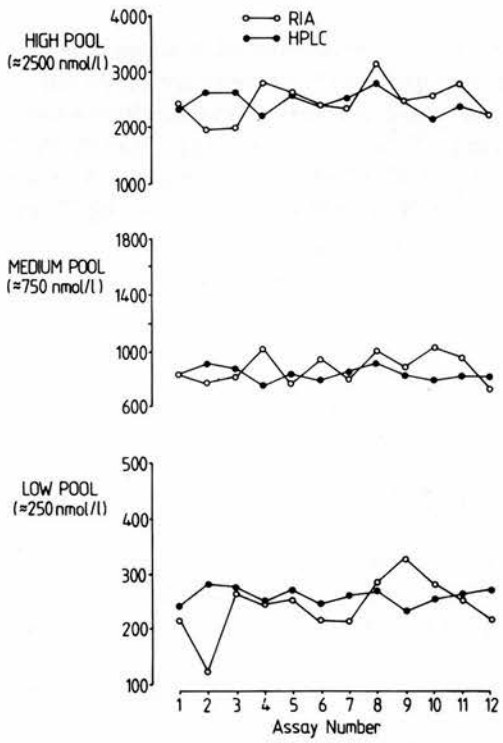

Fig. 3. Serotonin concentrations as measured in the spiked PPP pools in consecutive assays over a period of 2 mth. The pools were stored as 1 ml aliquots in sealed polypropylene microcentrifuge tubes at −20 °C.

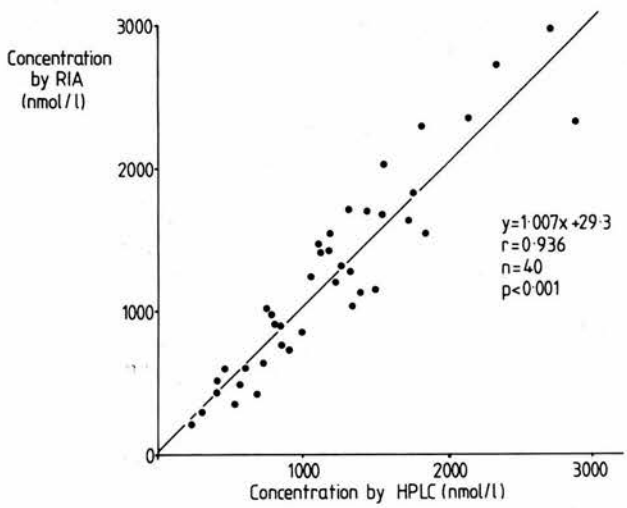

Fig. 4. Correlation of the RIA with the HPLC assay for human PRP samples from healthy volunteers and drug-free patients attending a hypertension clinic.

Validation of the RIA by HPLC

Figure 4 shows the regression equation for comparison of serotonin values measured by HPLC and RIA. The relatively small positive intercept (29 nmol/l) suggests that endogenous *N*-acetylserotonin is not present at concentrations sufficient to interfere with the RIA measurement. Of the normal subjects studied, PRP serotonin concentrations (mean ± SD) were 1 548 ± 541 nmol/l for the females ($n = 5$), and 1 404 ± 827 for the males ($n = 6$). This difference was not significant ($p > 0.5$, Student's t test).

Discussion

The stability of serotonin at room temperature and in the presence of atmospheric oxygen decreases at neutral or alkaline pH [22]. Consequently, we found it necessary to synthesise the *N*-succinamylserotonin under nitrogen, as exposure to the alkaline hydroxylamine used in cleaving the 5-*O*-succinyl moiety back to a 5-hydroxyl group otherwise resulted in destruction of the indole nucleus. Similarly, the preparation of the immunogen, was conducted under nitrogen. Acetylation by NAS instead of a non amino-specific acylating agent does not require an alkali hydrolysis step, and thus avoids the risk of oxidising the modified serotonin molecules. It seems possible that a similar approach could be used to develop assays for other unstable mono-amines.

The significant difference between the recovery of tritiated serotonin or the internal standard from PRP may be due to the impurities in the tracer, as it is not known exactly how the residual (< 10%) of radioactivity behaves, whether it is distributed throughout the sample, or is concentrated in the supernate, which would result in a falsely elevated recovery. Although it is assumed that the internal standard behaves in the same way as serotonin, and reflects the distribution of the analyte in the deproteinised sample, the fact that serotonin and the internal standard can be separated by HPLC underlines the point that they are not chemically identical and may indeed behave differently during the deproteinisation. If this is the case, and the [^{3}H]serotonin recovery is the true value, the internal standard recovery would overcompensate for loss of serotonin during deproteinisation, giving rise to elevated results by the HPLC assay. However, the small positive intercept in the correlation between the RIA and the HPLC assay does not support this, since the RIA needs no recovery correction figure, as the standards are diluted in the same medium and processed in the same way as the samples, therefore, respective recoveries should be similar.

The agreement between the RIA and the HPLC reference method shows that in human PRP the concentration of *N*-acetylserotonin is negligible compared with the concentration of serotonin, as the HPLC separates these two compounds, allowing them to be measured independently. This agrees with a previously published observation [16]. The present assay has been validated for human PRP, and the possible presence in other species of appreciable amounts of circulating *N*-acetylserotonin requires investigation. The assay could be used to measure *N*-acetylserotonin directly.

The sensitivity of our RIA is comparable both with that of previously published RIAs (2–10 nmol/l), which used either tritiated serotonin or gamma-emitting tracers, and also with that of HPLC with electrochemical detection and without extraction (approximately 2–5 nmol/l), although the precision in the RIA is poorer than the HPLC at the tested concentrations. This may be partly due to the use of an internal standard as a recovery marker in the HPLC assay, as this compensates for imprecision incorporated in sample preparation and analysis.

Of the previously published RIA methods [17–21], only one [19] was validated by comparison with an established method of serotonin measurement, but this RIA used tritiated serotonin as a tracer and a low working dilution of antiserum (1/100). The assay we have developed uses a ^{125}I tracer of high specific activity, and a high titre antiserum, and has been validated using an HPLC assay. The technical manipulations required for the RIA are no more difficult than for the HPLC assay, and up to 100 samples can easily be assayed in a working day, enabling the RIA to be adapted for routine laboratory use. It is anticipated that the development of this validated reproducible radioimmunoassay procedure for serotonin will lead to a better understanding of the role of platelet serotonin in the pathophysiology of disease states such as hypertension, migraine and depression.

Acknowledgements

Grateful thanks are due to Dr. P.L. Padfield and Research Nurses Marie Reid and Jane Corrie for their invaluable help in the collection of many of the samples used.

I.F. Gow was supported by a grant from Sandoz Pharmaceuticals.

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